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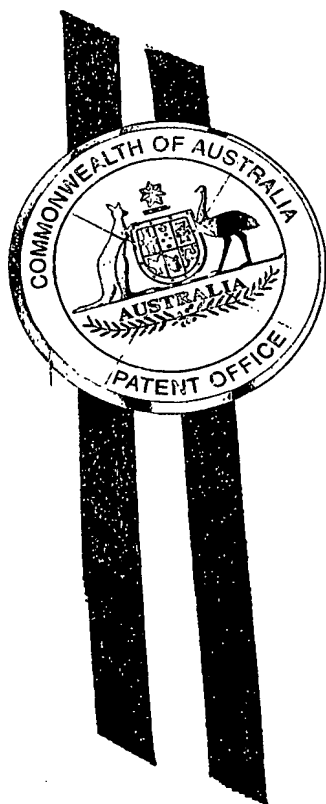
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AUSTRALIA

Patents Act 1990

Human Genetic Signatures Pty Ltd

PROVISIONAL SPECIFICATION

Invention Title:

Assay for Nucleic Acid Molecules

The invention is described in the following statement:

Technical Field

This invention relates to DNA hybridisation assays and to an improved oligonucleotide or intercalating nucleic acid (INA) assay. The invention relates particularly to methods for distinguishing specific base sequences including 5-methyl cytosine bases in DNA using these assays.

Background Art

A number of procedures were available for the detection of specific nucleic acid molecules. These procedures typically depend on sequence-dependent hybridisation between the target DNA and nucleic acid probes which may range in length from short oligonucleotides (20 bases or less) to sequences of many kilobases.

For direct detection, the target DNA is most commonly separated on the basis of size by gel electrophoresis and transferred to a solid support prior to hybridisation with a probe complementary to the target sequence (Southern and Northern blotting). The probe may be a natural nucleic acid or analogue such as INA or locked nucleic acid (LNA), PNA, HNA, ANA and MNA. The probe may be directly labelled (eg. with ^{32}P) or an indirect detection procedure may be used. Indirect procedures usually rely on incorporation into the probe of a "tag" such as biotin or digoxigenin and the probe is then detected by means such as enzyme-linked substrate conversion or chemiluminescence.

Another method for direct detection of nucleic acid that has been used widely is "sandwich" hybridisation. In this method, a capture probe is coupled to a solid support and the target DNA, in solution, is hybridised with the bound probe. Unbound target DNA is washed away and the bound DNA is detected using a second probe that hybridises to the target sequences. Detection may use direct or indirect methods as outlined above. The "branched DNA" signal detection system is an example that uses the sandwich hybridization principle (Urdea Ms Branched DNA signal amplification. Biotechnology 12: 926-928).

A rapidly growing area that uses nucleic acid hybridisation for direct detection of nucleic acid sequences is that of DNA micro-arrays (Young RA Biomedical discovery with DNA arrays. Cell 102: 9-15 (2000); Watson, New tools. A new breed of high tech detectives. Science 289:850-854 (2000)). In this process, individual nucleic acid species, that may range from oligonucleotides to longer sequences such as cDNA clones, were fixed to a solid

support in a grid pattern. A tagged or labelled nucleic acid population was then hybridised with the array and the level of hybridisation with each spot in the array is quantified. Most commonly, radioactively or fluorescently-labelled nucleic acids (eg. cDNAs) were used for hybridisation, though other detection systems were employed.

5 The most widely used method for amplification of specific sequences from within a population of nucleic acid sequences is that of polymerase chain reaction (PCR) (Dieffenbach C and Dveksler G eds. PCR Primer: A Laboratory Manual. Cold Spring Harbor Press, Plainview NY). In this amplification method, oligonucleotides, generally 15 to 30 nucleotides in length on complementary DNA strands and at either end of the DNA
10 region to be amplified, were used to prime DNA synthesis on denatured single-stranded DNA. Successive cycles of denaturation, primer hybridisation and DNA strand synthesis using thermostable DNA polymerases allows exponential amplification of the sequences between the primers. RNA sequences can be amplified by first copying using reverse transcriptase to produce a cDNA copy. Amplified DNA fragments can be detected by a
15 variety of means including gel electrophoresis, hybridisation with labelled probes, use of tagged primers that allow subsequent identification (eg. by an enzyme linked assay), use of fluorescently-tagged primers that give rise to a signal upon hybridisation with the target DNA (eg. Beacon and TaqMan systems).

20 As well as PCR, a variety of other techniques have been developed for detection and amplification of specific sequences. One example is the ligase chain reaction (Barany F Genetic disease detection and DNA amplification using cloned thermostable ligase. Proc. Natl. Acad. Sci. USA 88:189-193 (1991)).

25 Currently the method of choice to detect methylation changes in DNA, such as were found in the GSTP1 gene promoter region in prostate cancer, were dependent on PCR amplification of such sequences after bisulfite modification of DNA. In bisulfite-treated DNA, cytosines were converted to uracils (and hence amplified as thymines during PCR) while methylated cytosines were non-reactive and remain as cytosines (Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL and Paul CL. A genomic sequencing
30 protocol which yields a positive display of 5-methyl cytosine residues in individual DNA strands. PNAS 89: 1827-1831 (1992); Clark SJ, Harrison J, Paul CL and Frommer M. High sensitivity mapping of methylated cytosines. Nucleic Acids Res. 22: 2990-2997 (1994)). Thus (after bisulfite treatment) DNA containing 5-methyl cytosine bases will be different in

sequence from the corresponding unmethylated DNA. The Frommer et al 1992 results are the basis of the bisulfite method for sequencing 5-methyl cytosine residues in DNA. Several years later this assay was used as the basis of a PCR assay for the methylation status of CpG islands in US 5786146. Primers may be chosen to amplify non-selectively a region of the genome of interest to determine its methylation status, or may be designed to selectively amplify sequences in which particular cytosines were methylated (Herman JG, Graff JR, Myohanen S, Nelkin BD and Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG Islands. PNAS 93:9821-9826 (1996)).

Alternative methods for detection of cytosine methylation include digestion with restriction enzymes whose cutting is blocked by site-specific DNA methylation, followed by Southern blotting and hybridisation probing for the region of interest. This approach is limited to circumstances where a significant proportion (generally >10%) of the DNA is methylated at the site and where there is sufficient DNA, usually 10 µg, to allow for detection. Digestion with restriction enzymes whose cutting is blocked by site-specific DNA methylation, followed by PCR amplification using primers that flank the restriction enzyme site(s). This method can utilise smaller amounts of DNA but any lack of complete enzyme digestion for reasons other than DNA methylation can lead to false positive signals.

Several years ago, peptide nucleic acids (PNA) in which the entire deoxyribose-phosphate backbone has been exchanged with a structurally homomorphous uncharged polyamide backbone composed of N-(2-aminoethyl)glycine units have been developed (Ray A and Norden B. Peptide nucleic acid (PNA): its medical and biotechnical applications and for the future. FASEB J 14: 1041-1060 (2000)).

Methods have been developed utilizing PNA ligands for the sensitive and specific detection of DNA which do not require PCR amplification (WO 02/38801). Recently, a new DNA ligand, intercalating nucleic acid (INA), has been developed which has unique and useful properties.

The present inventors have developed new assays for detecting methylated cytosine residues in DNA using INA probes.

Disclosure of Invention

In a first aspect, the present invention provides a method for detecting the presence of a target DNA in a sample, the method comprising:

- (a) treating a sample containing DNA with an agent that modifies unmethylated cytosine;
- 5 (b) providing to the treated sample a detector ligand in the form of an intercalating nucleic acid (INA) capable of binding to a target region of DNA and allowing sufficient time for a detector ligand to bind to a target DNA; and
- (c) measuring binding of the detector ligand to DNA in the sample to determine the presence of the target DNA in a sample.

10 In a second aspect, the present invention provides a method for estimating extent of methylation of a target DNA in a sample, the method comprising:

- (a) treating a sample containing DNA with an agent that modifies unmethylated cytosine;
- (b) providing to the treated sample a detector ligand in the form of an intercalating nucleic acid (INA) capable of distinguishing between methylated and unmethylated cytosine
- 15 of DNA and allowing sufficient time for a detector ligand to bind to a target DNA; and
- (c) detecting binding of the detector ligand to DNA in the sample such that the degree or amount of binding is indicative of the extent of methylation of the target DNA.

In step (b), two detector ligands can be used where one ligand is capable of binding to a region of DNA that contains one or more methylated cytosines and the other ligand

20 capable of binding to a corresponding region of DNA that before treatment (step (a)) contained no methylated cytosines. As a sample can contain many copies of a target DNA, often the copies have different amounts of methylation. Accordingly, the ratio of binding of the two ligands will be proportional to the degree of methylation of that DNA target in the sample. The two ligands can be added together in the one test or can be added in

25 separate duplicate tests. Each ligand can contain a unique marker which can be detected concurrently or separately in the one test or have the same marker and detected individually in separate tests.

In a third aspect, the invention provides a method for detecting the presence of a target DNA in a sample, the method comprising:

- 30 (a) treating a sample containing DNA with an agent that modifies unmethylated cytosine;

(b) providing a support to which is bound a capture ligand which is capable of recognising a first part of a target DNA sequence;

(c) contacting the support with the treated sample for sufficient time to allow DNA to bind to a capture ligand such that target DNA in the sample binds to the support via the capture ligand;

(d) contacting the support with a detector ligand in the form of an intercalating nucleic acid (INA) capable of recognising a second part of the target DNA sequence and allowing sufficient time for a detector ligand to bind to a target DNA bound to a support; and

(e) measuring binding of the detector ligand to DNA bound to the support to determine the presence of the target DNA in the sample.

In a fourth aspect, the present invention provides a method for estimating extent of methylation of a target DNA in a sample, the method comprising:

(a) treating a sample containing DNA with an agent that modifies unmethylated cytosine;

(b) providing a support to which is bound a capture ligand which is capable of recognising a first part of a target DNA sequence;

(c) contacting the support with the treated sample for sufficient time to allow DNA to bind to a capture ligand such that target DNA in the sample binds to the support via the capture ligand;

(d) contacting the support with a detector ligand in the form of an intercalating nucleic acid (INA) capable of distinguishing between methylated and unmethylated cytosine of DNA such that the detector ligand binds to any target DNA on the support; and

(e) detecting binding of the detector ligand to the support such that the degree or amount of binding is indicative of the extent of methylation of the target DNA.

Preferably, the capture ligand is selected from intercalating nucleic acid (INA) probe, peptide nucleic acid (PNA) probe, LNA probe, HNA probe, ANA probe, MNA probe, oligonucleotide, modified oligonucleotide, single stranded DNA, RNA, aptamer, antibody, protein, peptide, a combination thereof, or chimeric versions thereof.

More preferably, the capture ligand is an INA probe, PNA probe or an oligonucleotide probe. Even more preferably, the capture ligand is an INA probe.

The support can be any suitable support such as a plastic materials, fluorescent beads, magnetic beads, synthetic or natural membranes, latex beads, polystyrene, column supports, glass beads or slides, nanotubes, fibres or other organic or inorganic supports. Preferably, the support is a magnetic-bead or a fluorescent bead.

5 The solid substrate is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs or microplates, or any other surface suitable for conducting an assay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically
10 adsorbing the molecule to the insoluble carrier.

In a preferred form, step (b) comprises a plurality of capture ligands arrayed on a solid support. The array may contain multiple copies of the same ligand so as to capture the same target DNA on the array or may contain a plurality of different ligands targeted to different DNA so as to capture a plurality of target DNA molecules on the array. Typically,
15 the array contains from about 10 to 200,000 capture ligands. It will be appreciated, however, that the array can have any number of capture ligands.

In one form, capture oligonucleotide probes, INA probes, or capture PNA probes can be placed on an array and used to capture bisulfite-treated DNA to measure methylated states of DNA. Array technology is well known and has been used to detect the presence of
20 genes or nucleotide sequences in untreated samples. The present invention, however, can extend the usefulness of array technology to provide valuable information on methylation states of many different sources of DNA.

The sample can be any biological sample such as stem cells, blood, urine, faeces, semen, cerebrospinal fluid, cells or tissue such as brain, colon, urogenital, lung, renal,
25 hematopoietic, breast, thymus, testis, ovary, or uterus, environmental samples, microorganisms including bacteria, virus, fungi, protozoan, viroid and the like. Stems cells include populations of cells containing true progenitor cells. This also applies to germ cell populations and also includes stem cells that fuse with somatic cells to form hybrid cells capable of adopting a particular phenotype.

30 In preferred forms, the sample is stem cells, blood, colorectal tissue, brain or prostate tissue.

Preferably, the modifying agent is capable of modifying unmethylated cytosine but not methylated cytosine. The agent is preferably selected from bisulfite, acetate and citrate. Preferably, the agent is sodium bisulfite and cytosine is modified to uracil.

The term "modifies" as used herein means the conversion of an unmethylated cytosine to another nucleotide which will distinguish the unmethylated from the methylated cytosine. Preferably, the agent modifies unmethylated cytosine to uracil. Preferably, the agent used for modifying unmethylated cytosine is sodium bisulfite, however, other agents that similarly modify unmethylated cytosine, but not methylated cytosine can also be used in the method of the invention. Sodium bisulfite (NaHSO_3) reacts readily with the 5,6-double bond of cytosine, but poorly with methylated cytosine. Cytosine reacts with the bisulfite ion to form a sulfonated cytosine reaction intermediate which is susceptible to deamination, giving rise to a sulfonated uracil. The sulfonate group can be removed under alkaline conditions, resulting in the formation of uracil. Thus all unmethylated cytosines will be converted to uracil while methylated cytosines will be protected from conversion so that ligands can be prepared that will recognise sequences containing cytosine or corresponding sequences containing uracil. The ratio of binding of the two probes can provide an accurate measure of the degree of methylation in a given DNA.

Importantly, in many situations there is no need to amplify the DNA to obtain the required information thus overcoming potential errors and resulting in a faster and more simple assay amenable to automation.

In a preferred form, the detector ligand is directed to a CpG- CpNpG-, or CNG- containing region of DNA, where N designates any one of the four possible bases A, T, C, or G. Preferably, the CpG- or CNG- containing region of DNA is in a regulatory region of a gene or an enhancer of any regulatory element or region including promoter, enhancer, oncogene, retro-element, mobile or mobilisable sequence or other regulatory element which activity is altered by environmental factors including chemicals, toxins, drugs, radiation, synthetic or natural compounds and microorganisms or other infectious agents such as viruses, bacteria, fungi and prions. For example, the promoter or regulatory element can be a tumour suppressor gene promoter, oncogene or any other element or region that may control or influence one or more genes implicated in a disease state or changing normal state such as aging.

The presence of methylated CpG- or CNG- containing region of DNA in a specimen can be indicative of a cell functional change. The change may be a proliferative disorder. It can include low grade astrocytoma, anaplastic astrocytoma, glioblastoma, medulloblastoma, colon cancer, lung cancer, renal cancer, leukemia, breast cancer, prostate cancer, endometrial cancer and neuroblastoma, or disturbances in normal cell division, differentiation or metabolism/catabolism of stem cell populations.

In order to assist in the reaction of the DNA modifying agent optional additives such as urea, methoxyamine and mixtures thereof can be added.

Step (b) is typically used to capture a DNA of interest which will be analysed for methylation in subsequent steps of the method. Thus, step (b) allows the capture and concentration of DNA of interest. Preferably a first INA or oligonucleotide probe is used in step (b).

In one preferred form, step (b) comprises a plurality of capture ligands arrayed on a solid support. The array may contain multiple copies of the same ligand so as to capture the same target DNA on the array for subsequent testing. Alternatively, the array may contain a plurality of different capture ligands targeted to different DNA molecules so as to capture many different target DNA samples on the array for subsequent testing.

In step (d), two detector ligands can be used where one ligand is capable of binding to a region of DNA that contains one or more methylated cytosines and the second ligand is capable of binding to a corresponding region of DNA that contains no methylated cytosines. A sample can contain many copies of a target DNA with the copies having different amounts of methylation. Accordingly, the ratio of binding of the two ligands will be proportional to the degree of methylation of that DNA target in the sample. The two ligands can be added together in the one test or can be added in separate duplicate tests. Each ligand can have an unique marker which can be detected concurrently or separately in the one test or have the same marker and detected individually in separate tests.

In order to detect binding of the detector ligand to a target DNA, preferably the ligand has a detectable label attached thereto. The presence of bound label being indicative of the extent of binding of the ligand. Suitable labels include fluorescence, radioactivity, enzyme, hapten and dendrimer.

The detector ligands used in the invention for detecting CpG- or CNG-containing DNA in a sample, after bisulfite modification, can specifically distinguish between untreated

DNA, methylated, and unmethylated DNA. Detector ligands in the form of oligonucleotide or PNA or INA probes for the non-methylated DNA preferably have a T or A in the 3' CG or CNG pair to distinguish it from the C retained in methylated DNA.

5 The probes of the invention were designed to be "substantially" complementary to one strand of the genomic locus to be tested and include the appropriate G or C nucleotides. This means that the primers should be sufficiently complementary to hybridize with a respective region of interest under conditions which allow binding. In other words, the probes should have sufficient complementarity with the 5' and 3' flanking sequences to hybridize therewith.

10 The INA probes of the invention may be prepared using any suitable method known to the art.

The methods according to the present invention relating to methylation states of target DNA can use any DNA sample, in purified or unpurified form, as the starting material, provided it contains, or is suspected of containing, the specific DNA sequence containing the
15 target region (usually CpG or CNG). In one preferred form, unamplified samples are used in the methods according to the present invention.

INA mixtures or specific INA molecules could be used in an amplification enrichment step prior to capture by the detector ligand. Single or large numbers of INAs could be used for specific or random amplification of bisulphite-treated DNA.

20 The DNA-containing specimen used for detection of methylated CpG or CNG may be from any source and may be extracted by a variety of techniques such as that described by Maniatis, et al (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y., pp 280, 281, 1982).

25 Where the DNA in the sample contains two strands, it is necessary to separate the strands of the DNA before it can be modified. Strand separation can be effected either as a separate step or simultaneously with chemical treatment. This strand separation can be accomplished using various suitable denaturing conditions, including physical, chemical, or enzymatic means, the word "denaturing" includes all such means. One physical method of separating DNA strands involves heating the DNA until it is denatured. Typical heat
30 denaturation may involve temperatures ranging from about 80° to 105°C for times ranging from about 1 to 10 minutes. Strand separation may also be induced by an enzyme from the class of enzymes known as helicases or by the enzyme RecA, which has helicase activity,

and in the presence of riboATP, is known to denature DNA. The reaction conditions suitable for strand separation of DNA with helicases were described by Kuhn Hoffmann-Berling (CSH-Quantitative Biology, 43:63, 1978) and techniques for using RecA were reviewed in C. Radding (Ann. Rev. Genetics, 16:405-437, 1982).

5 The detectable label may be fluorescent, or radioactive or contain a second label or marker in the form of a microsphere, or nanocrystal. The fluorescent or radioactive microsphere or nanocrystal may be covalently bound to the capture or detector ligand.

Preferably the specificity of hybridization to target DNA is used to discriminate between methylated cytosines and unmethylated cytosines.

10 Many suitable fluorochromes that bind to DNA, some selective for single-stranded DNA, and that differ in their excitation and emission wavelengths were known. The detection system could also be an enzyme carrying a positively charged region that will selectively bind to the DNA and that can be detected using an enzymatic assay, or a positively charged radioactive molecule that binds selectively to the captured DNA. The
15 suitable entity may also be core/shell CdSe/ZnS semiconductor nanocrystals (gerion et al 2002 J Am Chem Soc 24:7070-7074).

Using INA probes as one of the ligands in this procedure has very significant advantages over the use of oligonucleotide probes. INA binding reaches equilibrium faster and exhibits greater sequence specificity and, as INAs carry one or more intercalating
20 groups, they bind the target DNA molecules with a higher binding coefficient than other ligands such as oligonucleotides or PNAs. The binding characteristics can be modified by choosing different numbers of intercalating groups to ad to the INA.

As the invention can use direct detection methods, they give a true and accurate measure of the amount of a target DNA in a sample. The methods were not confounded by
25 potential bias inherent in methods that rely for signal amplification on processes such as PCR, where the enzymes commonly used in such procedures can introduce systematic bias through differential rates of amplification of different sequences.

In a fifth aspect, the present invention provides a method for detecting a methylated CpG- or CNG-containing DNA, the method comprising:

30 (a) treating a sample containing DNA with bisulfite to modify unmethylated cytosine to uracil in the DNA;

- (b) providing to the treated sample a detector INA ligand capable of distinguishing between methylated and unmethylated cytosine of DNA; and
- (c) detecting the methylated DNA based on the presence or absence of binding of the detector INA ligand.

5

In one preferred form, the method comprises:

- (a) treating a DNA-containing specimen with bisulfite to modify unmethylated cytosine to uracil,
- (b) providing to the treated sample a detector ligand capable of binding to a methylated CpG- or CNG-containing DNA but not to a corresponding unmethylated CpG- or
- 10 CNG-containing DNA; and
- (c) detecting binding of the ligand to DNA in the sample such that binding is indicative of methylation of the DNA.

Preferably, the detector ligand is an INA probe.

15 In a preferred form, the invention provides a method for estimating extent of methylation of a target DNA in a sample, the method comprising:

- (a) treating a sample containing DNA with bisulfite to modify unmethylated cytosine to uracil;
- (b) providing a solid support in the form of a magnetic bead to which is bound a capture INA or oligonucleotide ligand which is capable of recognising a first part of a target
- 20 DNA sequence;
- (c) contacting the support with the treated sample suspected of containing the target DNA such that target DNA in the sample binds to the support via the capture ligand;
- (d) contacting the support with a detector INA ligand capable of distinguishing between methylated and unmethylated cytosine of DNA; and
- 25 (e) determining the extent of methylation of the DNA bound to the support by measuring the amount of bound detector ligand.

30 In a sixth aspect, the present invention relates to use of an agent that modifies unmethylated cytosine but not methylated cytosine and one or more ligands, preferably one or more INA probes, capable of distinguishing between methylated and unmethylated cytosine of DNA in methods for assaying methylation of target DNA.

There are a number of detector systems and instruments available for detecting or measuring fluorescence or radioactivity. Improvements and advancement in instrumentation are being made by a number of manufacturers. It will be appreciated that many different measuring instruments can be used for the present invention. For example, Multi Photon

5 Detection is a proprietary system for the detection of ultra low amounts of selected radioisotopes. It is 1000 fold more sensitive than existing methods. It has a sensitivity of 1000 atoms of iodine 125, with quantitation of zeptomole amounts of biomaterials. It requires less than 1 picroCurie of isotope which is 100 times less activity than in a glass of water. A family of MPD instruments already exists for measuring radioactivity in a sample. 10 They consist of instruments that are configured for 96 well, 384 well and higher. MPD uses coincident multichannel detection of photons coupled with computer controlled electronics to selectively count only those photons that are compatible with an operator-selected radioisotope. As many different isotopes can be used, this is a multicolor system. The MPD imager system is at least 100 fold more sensitive than a phosphor imager. Such 15 instrumentation would be particularly suitable in the detection part of the present invention where ligands or supports are made radioactive.

Beads containing capture or detector ligands bound thereto can be processed or measured by cell sorters which measure fluorescence. Examples of suitable instruments include flow cytometers and modified versions thereof.

20 The methods according to the present invention are particularly suitable for scaling up and automation for processing many samples.

Notwithstanding the above, the methods described can be used in conjunction with such amplification procedures if it is necessary to amplify limiting amounts of DNA in order provide enough material for detection.

25 **Methylated DNA:** In a particular adaptation as detailed in the present invention, the methods can be used to distinguish the presence of methylated cytosines in DNA that has been treated with sodium bisulfite. As cytosines were converted to uracils while methyl cytosines remain unreacted, the sequence of bisulfite-treated DNA derived from methylated and unmethylated molecules is different. By choosing a specific INA ligand (4 to 100 30 residues long, preferably 15 ± 5 residues long) to selected target regions the specificity of hybridisation can be used to discriminate between methylated cytosines at CpG or CNG sites (which remain as cytosines) and unmethylated CpG or CNG sites where the cytosine is

converted to uracil, while ensuring that only molecules in which cytosines that were not in CpG or CNG sites have fully reacted and been converted to uracils were assessed.

Methylated cytosines at other sites can similarly be detected. Appropriate INA probes can be used as controls to identify the presence of molecules that have not reacted completely with bisulfite (one or more cytosines not converted to uracil). It will be appreciated, however, that other ligands which can differentiate between the methylation states of DNA can be used in a similar manner.

The methods were amenable for use in a variety of formats including multiwell plates, micro-arrays, fiber optic arrays and particles in suspension. The appropriate selection of specific ligands for use in an array format can allow for the simultaneous determination of the methylation state of individual cytosines in multiple target regions.

Polymorphism/mutation epimutation detection: The methods according to the present invention can be applied to the discrimination of different alleles of a gene where the sequence of the capture ligand and/or the detector ligand will match with one allele but mismatch with the other.

DNA Quantification: By using the methods according to the present invention, it is possible to directly determine within a DNA population the proportion of molecules having one sequence versus another at a particular region. This can be done by coupling ligands representing the alternate forms of the sequence to supports such as microspheres charged with differently coloured fluorochromes, nanocrystals or radioactive molecules. Such differences in sequence may be differences in the original base sequence of the gene or differences in base sequence in bisulfite-treated DNA that were due to differences in methylation in the original DNA.

Cell quantification: The methods can be applied to determining the ratio of cells in a population (such as in cancer and normal cells) that differ in base sequence at a particular site in the genome.

Variations: The methods were amenable for use in a variety of formats including multiwell plates, micro-arrays, fiber optic arrays and particles in suspension. The appropriate selection of specific INA probes for use in an array format can allow for the simultaneous determination of the presence of different DNA sequences, eg. for the determination of the methylation state of individual cytosines in multiple target regions.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following drawings and examples.

15 Brief Description of the Drawings

Figure 1 shows range of different backbone monomer units of nucleotides and nucleotide analogues, and how they are connected to the nucleobases via linkers that are attached at one or two positions of the backbone monomer unit.

Figure 2 shows general structures of suitable acyclic backbone monomers.

Figure 3 shows suitable acyclic backbone monomer units.

Figure 4 shows preferred intercalators.

Figure 5 shows preferred linker chains.

Figure 6 shows preferred INA molecules (intercalator pseudonucleotides).

Figure 7 shows an INA (intercalator pseudonucleotide) preparation scheme.

Figure 8 shows alternative synthesis procedure for 3-(1-Pyrenylmethoxy)-propane-1,2-diol.

Figure 9 shows scheme for synthesis of the 2-O- phosphoramidite of 1-O-4,4'-dimethoxytrityl-4-O-(9-antracenylmethyl)-1,2,4-butanetriol

Figure 10 shows scheme for synthesis of the Phosphoramidite of (S)-1-(4,4'-dimethoxytriphenylmethyloxy)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-2-butanol (V)

Figure 11 shows a general overview of sandwich signal amplification methodology using INA probes for detection of methylated DNA.

Figure 12 shows a general overview of sandwich signal amplification methodology using INA probes and magnetic beads for detection of methylated DNA.

5 Figure 13 shows part of the nucleic acid sequence of the GSTP1 gene and methylation states of that gene region.

Mode(s) for Carrying Out the Invention

DEFINITIONS

10 Nucleic acids

The term "nucleic acid" covers the naturally occurring nucleic acids, DNA and RNA. The term "nucleic acid analogues" covers derivatives of the naturally occurring nucleic acids, DNA and RNA, as well as synthetic analogues of naturally occurring nucleic acids. Synthetic analogues comprise one or more nucleotide analogues. The term nucleotide
15 analogue includes all nucleotide analogues capable of being incorporated into a nucleic acid backbone and capable of specific base-pairing (see below), essentially like naturally occurring nucleotides.

Hence the terms "nucleic acid" or "nucleic acid analogues" designate any molecule which essentially consists of a plurality of nucleotides and/or nucleotide analogues and/or
20 intercalator pseudonucleotides. Nucleic acids or nucleic acid analogues useful for the present invention may comprise a number of different nucleotides with different backbone monomer units.

Preferably, single strands of nucleic acids or nucleic acid analogues are capable of hybridising with an substantially complementary single stranded nucleic acid and/or nucleic
25 acid analogue to form a double stranded nucleic acid or nucleic acid analogue. More preferably such a double stranded analogue is capable of forming a double helix. Preferably, the double helix is formed due to hydrogen bonding, more preferably, the double helix is a double helix selected from the group consisting of double helices of A form, B form, Z form and intermediates thereof.

30 Hence, nucleic acids and nucleic acid analogues useful for the present invention include, but is not limited to DNA, RNA, LNA, PNA, MNA, ANA, HNA and mixtures thereof

and hybrids thereof, as well as phosphorous atom modifications thereof, such as but not limited to phosphorothioates, methyl phospholates, phosphoramidites, phosphorodithiates, phosphoroselenoates, phosphotriesters and phosphoboranoates. In addition non-phosphorous containing compounds may be used for linking to nucleotides such as but not limited to methyliminomethyl, formacetate, thioformacetate and linking groups comprising amides. In particular nucleic acids and nucleic acid analogues may comprise one or more intercalator pseudonucleotides.

Within this context "mixture" is meant to cover a nucleic acid or nucleic acid analogue strand comprising different kinds of nucleotides or nucleotide analogues. Furthermore, within this context, "hybrid" is meant to cover nucleic acids or nucleic acid analogues comprising one strand which comprises nucleotide or nucleotide analogue with one or more kinds of backbone and another strands which comprises nucleotide or nucleotide analogue with different kinds of backbone.

By HNA is meant nucleic acids as for example described by Van Aetschot et al., 1995. By MNA is meant nucleic acids as described by Hossain et al, 1998. ANA refers to nucleic acids described by Allert et al, 1999. LNA may be any LNA molecule as described in WO 99/14226 (Exiqon), preferably, LNA is selected from the molecules depicted in the abstract of WO 99/14226. More preferably, LNA is a nucleic acid as described in Singh et al, 1998, Koshkin et al, 1998 or Obika et al., 1997. PNA refers to peptide nucleic acids as for example described by Nielsen et al, 1991.

The term nucleotide designates the building blocks of nucleic acids or nucleic acid analogues and the term nucleotide covers naturally occurring nucleotides and derivatives thereof as well as nucleotides capable of performing essentially the same functions as naturally occurring nucleotides and derivatives thereof. Naturally occurring nucleotides comprise deoxyribonucleotides comprising one of the four main nucleobases adenine (A), thymine (T), guanine (G) or cytosine (C), and ribonucleotides comprising one of the four nucleobases adenine (A), uracil (U), guanine (G) or cytosine (C). In addition to the main or common bases above, other less common naturally occurring bases which can exist in some nucleic acid molecules include 5-methyl cytosine (met-C) and 6-methyl adenine (met-A).

Nucleotide analogues may be any nucleotide like molecule that is capable of being incorporated into a nucleic acid backbone and capable of specific base-pairing.

Non-naturally occurring nucleotides includes, but is not limited to the nucleotides comprised within DNA, RNA, PNA, HNA, MNA, ANA, LNA, CNA, CeNA, TNA, (2'-NH)-TNA, (3'-NH)-TNA, α -L-Ribo-LNA, α -L-Xylo-LNA, β -D-Xylo-LNA, α -D-Ribo-LNA, [3.2.1]-LNA, Bicyclo-DNA, 6-Amino-Bicyclo-DNA, 5-epi-Bicyclo-DNA, α -Bicyclo-DNA, Tricyclo-DNA, Bicyclo[4.3.0]-DNA, Bicyclo[3.2.1]-DNA, Bicyclo[4.3.0]amide-DNA, β -D-Ribopyranosyl-NA, α -L-Lyxopyranosyl-NA, 2'-R-RNA, α -L-RNA or α -D-RNA, β -D-RNA.

The function of nucleotides and nucleotide analogues is to be able to interact specifically with complementary nucleotides via hydrogen bonding of the nucleobases of the complementary nucleotides as well as to be able to be incorporated into a nucleic acid or nucleic acid analogue. Naturally occurring nucleotide, as well as some nucleotide analogues are capable of being enzymatically incorporated into a nucleic acid or nucleic acid analogue, for example by RNA or DNA polymerases. However, nucleotides or nucleotide analogues may also be chemically incorporated into a nucleic acid or nucleic acid analogue.

Furthermore nucleic acids or nucleic acid analogues may be prepared by coupling two smaller nucleic acids or nucleic acid analogues to another, for example this may be done enzymatically by ligases or it may be done chemically.

Nucleotides or nucleotide analogues comprise a backbone monomer unit and a nucleobase. The nucleobase may be a naturally occurring nucleobase or a derivative thereof or an analogue thereof capable of performing essentially the same function. The function of a nucleobase is to be capable of associating specifically with one or more other nucleobases via hydrogen bonds. Thus it is an important feature of a nucleobase that it can only form stable hydrogen bonds with one or a few other nucleobases, but that it can not form stable hydrogen bonds with most other nucleobases usually including itself. The specific interaction of one nucleobase with another nucleobase is generally termed "base-pairing".

The base pairing results in a specific hybridisation between predetermined and complementary nucleotides. Complementary nucleotides are nucleotides that comprise nucleobases that are capable of base-pairing.

Of the common naturally occurring nucleobases, adenine (A) pairs with thymine (T) or uracil (U); and guanine (G) pairs with cytosine (C). Accordingly, a nucleotide comprising

A is complementary to a nucleotide comprising either T or U, and a nucleotide comprising G is complementary to a nucleotide comprising C.

Nucleotides may further be derivatised to comprise an appended molecular entity. The nucleotides can be derivatised on the nucleobases or on the backbone monomer unit.

5 Preferred sites of derivatisation on the bases include the 8-position of adenine, the 5-position of uracil, the 5- or 6-position of cytosine, and the 7-position of guanine. The heterocyclic modifications can be grouped into three structural classes: Enhanced base stacking, additional hydrogen bonding, and the combination of these classes. Modifications that enhance base stacking by expanding the π -electron cloud of the planar systems are

10 represented by conjugated, lipophilic modifications in the 5-position of pyrimidines and the 7-position of 7-deaza-purines. Substitutions in the 5-position of pyrimidines modifications include propynes, hexynes, thiazoles and simply a methyl group; and substituents in the 7-position of 7-deaza purines include iodo, propynyl, and cyano groups. It is also possible to modify the 5-position of cytosine from propynes to five-membered heterocycles and to

15 tricyclic fused systems, which emanate from the 4- and 5-position (cytosine clamps). A second type of heterocycle modification is represented by the 2-amino-adenine where the additional amino group provides another hydrogen bond in the A-T base pair, analogous to the three hydrogen bonds in a G-C base pair. Heterocycle modifications providing a combination of effects are represented by 2-amino-7-deaza-7-modified adenine and the

20 tricyclic cytosine analog having an ethoxyamino functional group of heteroduplexes. Furthermore, N2-modified 2-amino adenine modified oligonucleotides are among commonly modifications. Preferred sites of derivatisation on ribose or deoxyribose moieties are modifications of non-connecting carbon positions C-2' and C-4', modifications of connecting carbons C-1', C-3' and C-5', replacement of sugar oxygen, O-4', anhydro sugar

25 modifications (conformational restricted), cyclosugar modifications (conformational restricted), ribofuranosyl ring size change, connection sites – sugar to sugar, (C-3' to C-5'/C-2' to C-5'), hetero-atom ring – modified sugars and combinations of above modifications. However, other sites may be derivatised, as long as the overall base pairing specificity of a nucleic acid or nucleic acid analogue is not disrupted. Finally, when the backbone monomer

30 unit comprises a phosphate group, the phosphates of some backbone monomer units may be derivatised.

Oligonucleotide or oligonucleotide analogue as used herein are molecules essentially consisting of a sequence of nucleotides and/or nucleotide analogues and/or

intercalator pseudonucleotides. Preferably oligonucleotide or oligonucleotide analogue comprises 5 to 100 individual nucleotides. Oligonucleotide or oligonucleotide analogues may comprise DNA, RNA, LNA, 2'-O-methyl RNA, PNA, ANA, HNA and mixtures thereof, as well as any other nucleotide and/or nucleotide analogue and/or intercalator
 5 pseudonucleotide.

Corresponding nucleic acids

Nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotides analogues are considered to be corresponding when they are capable of hybridising.
 10 Preferably corresponding nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotides analogues are capable of hybridising under low stringency conditions, more preferably corresponding nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotides analogues are capable of hybridising under medium stringency conditions, more preferably corresponding nucleic acids, nucleic acid analogues, oligonucleotides or
 15 oligonucleotides analogues are capable of hybridising under high stringency conditions.

High stringency conditions as used herein shall denote stringency as normally applied in connection with Southern blotting and hybridisation as described e.g. by Southern E. M., 1975, J. Mol. Biol. 98:503-517. For such purposes it is routine practise to include steps of prehybridization and hybridization. Such steps are normally performed using
 20 solutions containing 6x SSPE, 5% Denhardt's, 0.5% SDS, 50% formamide, 100 µg/ml denatured salmon testis DNA (incubation for 18 hrs at 42°C), followed by washing with 2x SSC and 0.5% SDS (at room temperature and at 37°C), and washing with 0.1x SSC and 0.5% SDS (incubation at 68°C for 30 min), as described by Sambrook et al., 1989, in "Molecular Cloning/A Laboratory Manual", Cold Spring Harbor, which is incorporated herein
 25 by reference.

Medium stringency conditions as used herein shall denote hybridisation in a buffer containing 1 mM EDTA, 10mM Na₂HPO₄·H₂O, 140 mM NaCl, at pH 7.0. Preferably, around 1.5 µM of each nucleic acid or nucleic acid analogue strand is provided. Alternatively medium stringency may denote hybridisation in a buffer containing 50 mM KCl, 10 mM
 30 TRIS-HCl (pH 9.0), 0.1% Triton X-100, 2 mM MgCl₂.

Low stringency conditions denote hybridisation in a buffer constituting 1 M NaCl, 10 mM Na₃PO₄ at pH 7.0.

Alternatively, corresponding nucleic acids, nucleic acid analogues, oligonucleotides or oligonucleotides, nucleic acid analogues, oligonucleotides or oligonucleotides substantially complementary to each other over a given sequence, such as more than 70% complementary, for example more than 75% complementary, such as more than 80% complementary, for example more than 85% complementary, such as more than 90% complementary, for example more than 92% complementary, such as more than 94% complementary, for example more than 95% complementary, such as more than 96% complementary, for example more than 97% complementary.

Preferably the given sequence is at least 10 nucleotides long, such as at least 15 nucleotides, for example at least 20 nucleotides, such as at least 25 nucleotides, for example at least 30 nucleotides, such as between 10 and 500 nucleotides, for example between 10 and 100 nucleotides long, such as between 10 and 50 nucleotides long. More preferably corresponding oligonucleotides or oligonucleotides analogues are substantially complementary over their entire length.

Cross-hybridisation

The term cross-hybridisation covers unintended hybridisation between at least two nucleic acids or nucleic acid analogues. Hence the term cross-hybridization may be used to describe the hybridisation of for example a nucleic acid probe or nucleic acid analogue probe sequence to other nucleic acid sequences or nucleic acid analogue sequences than its intended target sequence.

Often cross-hybridization occurs between a probe and one or more corresponding non-target sequences, even though these have a lower degree of complementarity than the probe and its corresponding target sequence. This unwanted effect could be due to a large excess of probe over target and/or fast annealing kinetics. Cross-hybridization also occurs by hydrogen bonding between few nucleobase pairs, e.g. between primers in a PCR reaction, resulting in primer dimer formation and/ or formation of unspecific PCR products.

Nucleic acids comprising one or more nucleotide analogues with high affinity for nucleotide analogues of the same type tend to form dimer or higher order complexes based on base pairing. Probes comprising nucleotide analogues such as, but not limited to, LNA, 2'-O-methyl RNA and PNA generally have a high affinity for hybridising to other oligonucleotide analogues comprising backbone monomer units of the same type. Hence

even though individual probe molecules only have a low degree of complementarity they tend to hybridize.

Self-hybridisation

5 The term self-hybridisation covers the process wherein a nucleic acid or nucleic acid
analogue molecule anneals to itself by folding back on itself, generating a secondary
structure like for example a hairpin structure, or one molecule binding to another identical
10 molecule leading to aggregation of the molecules. In most applications it is of importance to
avoid self-hybridization. Furthermore, self hybridization can also increase background signal
and importantly decrease the sensitivity of molecular biological methods or assays. The
generation of secondary structures may inhibit hybridisation with desired nucleic acid target
sequences. This is undesired in most assays for example when the nucleic acid or nucleic
acid analogue is used as primer in PCR reactions or as fluorophore/ quencher labelled
15 probe for exonuclease assays. In both assays, self-hybridisation will inhibit hybridization to
the target nucleic acid and additionally the degree of fluorophore quenching in the
exonuclease assay is lowered.

Nucleic acids comprising one or more nucleotide analogues with high affinity for
nucleotide analogues of the same type tend to self-hybridize. Probes comprising nucleotide
analogues such as, but not limited to, LNA, 2'-O-methyl RNA and PNA generally have a high
20 affinity for self-hybridising. Hence even though individual probe molecules only have a low
degree of self-complementary they tend to self-hybridize.

Melting temperature

25 Melting of nucleic acids refer to the separation of the two strands of a double-
stranded nucleic acid molecule. The melting temperature (T_m) denotes the temperature in
degrees celsius at which 50% helical (hybridized) versus coil (unhybridized) forms are
present.

A high melting temperature is indicative of a stable complex and accordingly of a
high affinity between the individual strands. Similarly, a low melting temperature is indicative
30 of a relatively low affinity between the individual strands. Accordingly, usually strong
hydrogen bonding between the two strands results in a high melting temperature.

Furthermore, intercalation of an intercalator between nucleobases of a double stranded nucleic acid may also stabilise double stranded nucleic acids and accordingly result in a higher melting temperature.

In addition, the melting temperature is dependent on the physical/chemical state of the surroundings. For example the melting temperature is dependent on salt concentration and pH.

The melting temperature may be determined by a number of assays, for example it may be determined by using the UV spectrum to determine the formation and breakdown (melting) of hybridisation.

Intercalating nucleic acid (INA) or intercalator pseudonucleotide

Intercalating nucleic acids (INA) are also termed intercalator pseudonucleotides in this specification.

Pseudonucleotides or polynucleotide analogues comprising intercalators and having one or more of the following desirable characteristics:

Intercalate into the double helix at a predetermined position;

- I. Substantially increase the affinity for DNA;
- II. Inhibit or decrease self and cross hybridisation;
- III. Discriminate between different nucleic acids, such as RNA and DNA;
- IV. Substantially increase the specificity of hybridisation;
- V. Increase nuclease stability;
- VI. Enhance strand invasion significantly;
- VII. Show a change in fluorescence intensity upon hybridisation.

An intercalator pseudonucleotide has the general structure:



wherein

X is a backbone monomer unit capable of being incorporated into the backbone of a nucleic acid or nucleic acid analogue,

Q is an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of DNA; and

5 Y is a linker moiety linking the backbone monomer unit and the intercalator.

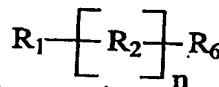
More preferably an intercalator pseudonucleotide has the general structure:



10 wherein

X is a backbone monomer unit capable of being incorporated into the backbone of a

nucleic acid or nucleic acid analogue of the general formula,



wherein $n = 1$ to 6

15 R_1 is a trivalent or pentavalent substituted phosphor atom,

R_2 is individually selected from an atom capable of forming at least two bonds, R_2 optionally being individually substituted, and

R_6 is a protecting group;

20 Q is an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of DNA; and

Y is a linker moiety linking any of R_2 of the backbone monomer unit and the intercalator; and

wherein the total length of Q and Y is in the range from about 7 Å to 20 Å.

25 When the intercalator is pyrene, for example, the total length of Q and Y is in the range from about 9 Å to 13 Å, preferably from about 9 Å to 11 Å.

By the term "incorporated into the backbone of a nucleic acid or nucleic acid analogue" is meant that the intercalator pseudonucleotide may be inserted into a sequence of nucleic acids and/or nucleic acid analogues.

By the term "flat conjugated system" is meant that substantially all atoms included in the conjugated system are located in one plane.

By the term "essentially flat conjugated system" is meant that at most 20% of all atoms included in the conjugated system are not located in the one plane at any time.

By the term "conjugated system" is meant a structural unit containing chemical bonds with overlap of atomic p orbitals of three or more adjacent atoms (Gold et al., 1987. Compendium of Chemical Terminology, Blackwell Scientific Publications, Oxford, UK).

Co-stacking is used in short for coaxial stacking. Coaxial stacking is an energetically favorable structure where flat molecules align on top of each other (flat side against flat side) along a common axis in a stack-like structure. Co-stacking requires interaction between two pi-electron clouds of individual molecules. In the case of intercalator pseudonucleotides, co-stacking with nucleobases in a duplex, preferably there is an interaction with a pi electron system on an opposite strand, more preferably there is interaction with pi electron systems on both strands. Co-stacking interactions are found both inter- and intra-molecularly. For example nucleic acids adopt a duplex structure to allow nucleobase co-stacking.

20 **Backbone monomer unit**

Any suitable backbone monomer unit may be employed. The backbone monomer unit comprises the part of an intercalator pseudonucleotide that may be incorporated into the backbone of an oligonucleotide or an oligonucleotide analogue. In addition, the backbone monomer unit may comprise one or more leaving groups, protecting groups and/or reactive groups, which may be removed or changed in any way during synthesis or subsequent to synthesis of an oligonucleotide or oligonucleotide analogue comprising the backbone monomer unit.

The term 'backbone monomer unit' only includes the backbone monomer unit *per se* and it does not include, for example, a linker connecting a backbone monomer unit to an intercalator. Hence, the intercalator as well as the linker is not part of the backbone monomer unit.

Accordingly, backbone monomer units only include atoms, wherein the monomer is incorporated into a sequence, are selected from the group consisting of

atoms which are capable of forming a linkage to the backbone monomer unit of a neighboring nucleotide; or

5 atoms which at least at two sites are connected to other atoms of the backbone monomer unit; or

atoms which at one site is connected to the backbone monomer unit and otherwise is not connected with other atoms.

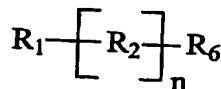
10 Backbone monomer unit atoms are thus defined as the atoms involved in the direct linkage (shortest path) between the backbone Phosphor-atoms of neighbouring nucleotides, when the monomer is incorporated into a sequence, wherein the neighbouring nucleotides are naturally occurring nucleotides.

The backbone monomer unit may be any suitable backbone monomer unit. The backbone monomer unit may for example be selected from the group consisting of the
15 backbone monomer units of DNA, RNA, PNA, HNA, MNA, ANA, LNA, CNA, CeNA, TNA, (2'-NH)-TNA, (3'-NH)-TNA, α -L-Ribo-LNA, α -L-Xylo-LNA, β -D-Xylo-LNA, α -D-Ribo-LNA, [3.2.1]-LNA, Bicyclo-DNA, 6-Amino-Bicyclo-DNA, 5-epi-Bicyclo-DNA, α -Bicyclo-DNA, Tricyclo-DNA, Bicyclo[4.3.0]-DNA, Bicyclo[3.2.1]-DNA, Bicyclo[4.3.0]amide-DNA, β -D-Ribopyranosyl-NA, α -L-Lyxopyranosyl-NA, 2'-R-RNA, α -L-RNA or α -D-RNA, β -D-RNA.

20 Figure 1 shows a range of different backbone monomer units of nucleotides and nucleotide analogues, and how they are connected to the nucleobases via linkers that are attached at one or two positions of the backbone monomer unit.

The backbone monomer unit of LNA (locked nucleic acid) is a sterically restricted DNA backbone monomer unit, which comprises an intramolecular bridge that restricts the
25 usual conformational freedom of a DNA backbone monomer unit. LNA may be any LNA molecule as described in WO 99/14226 (Exiqon). Preferred LNA comprises a methyl linker connecting the 2'-O position to the 4'-C position, however other LNA's such as LNA's wherein the 2' oxy atom is replaced by either nitrogen or sulphur are also comprised within the present invention.

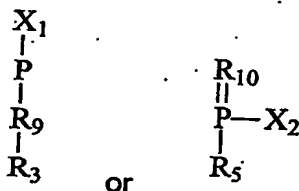
The backbone monomer unit of intercalator pseudonucleotides preferably have the general structure before being incorporated into an oligonucleotide and/or nucleotide analogue:



wherein

$n = 1$ to 6 , preferably $n = 2$ to 6 , more preferably $n = 3$ to 6 , more preferably $n = 2$ to 5 , more preferably $n = 3$ to 5 , more preferably $n = 3$ to 4 ;

R_1 is a trivalent or pentavalent substituted phosphor atom, preferably R_1 is



wherein

R_2 may individually be selected from an atom capable of forming at least two bonds, the atom optionally being individually substituted, preferably R_2 is individually selected from O, S, N, C, P, optionally individually substituted. By the term "individually" is meant that R_2 can represent one, two or more different groups in the same molecule. The bonds between two R_2 may be saturated or unsaturated or a part of a ring system or a combination thereof. Each R_2 may individually be substituted with any suitable substituent, such as a substituent selected from H, lower alkyl, C2-C6 alkenyl, C6-C10 aryl, C7-C11 arylmethyl, C2-C7 acyloxymethyl, C3-C8 alkoxycarbonyloxymethyl, C7-C11 aryloxyloxymethyl, C3-C8 S-acyl-2-thioethyl.

An "alkyl" group refers to an optionally substituted saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, the alkyl group has 1 to 25 carbons and contains no more than 20 heteroatoms. More preferably, it is a lower alkyl of from 1 to 12 carbons, more preferably 1 to 6 carbons, more preferably 1 to 4

carbons. Heteroatoms are preferably selected from the group consisting of nitrogen, sulfur, phosphorus, and oxygen.

5 An "alkenyl" group refers to an optionally substituted hydrocarbon containing at least one double bond, including straight-chain, branched-chain, and cyclic alkenyl groups, all of which may be optionally substituted. Preferably, the alkenyl group has 2 to 25 carbons and contains no more than 20 heteroatoms. More preferably, it is a lower alkenyl of from 2 to 12 carbons, more preferably 2 to 4 carbons. Heteroatoms are preferably selected from the group consisting of nitrogen, sulfur, phosphorus, and oxygen.

10 An "alkynyl" group refers to an optionally substituted unsaturated hydrocarbon containing at least one triple bond, including straight-chain, branched-chain, and cyclic alkynyl groups, all of which may be optionally substituted. Preferably, the alkynyl group has 2 to 25 carbons and contains no more than 20 heteroatoms. More preferably, it is a lower alkynyl of from 2 to 12 carbons, more preferably 2 to 4 carbons. Heteroatoms are preferably selected from the group consisting of nitrogen, sulfur, phosphorus, and oxygen.

15 An "aryl" refers to an optionally substituted aromatic group having at least one ring with a conjugated pi electron system and includes carbocyclic aryl, heterocyclic aryl, bi-aryl, and tri-aryl groups. Examples of aryl substitution substituents include alkyl, alkenyl, alkynyl, aryl, amino, substituted amino, carboxy, hydroxy, alkoxy, nitro, sulfonyl, halogen, thiol and aryloxy.

20 A "carbocyclic aryl" refers to an aryl where all the atoms on the aromatic ring are carbon atoms. The carbon atoms are optionally substituted as described above for an aryl. Preferably, the carbocyclic aryl is an optionally substituted phenyl.

25 A "heterocyclic aryl" refers to an aryl having 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen. Examples of heterocyclic aryls include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, and imidazolyl. The heterocyclic aryl is optionally substituted as described above for an aryl.

30 The substituents on two or more R_2 may alternatively join to form a ring system, such as any of the ring systems as defined above. Preferably R_2 is substituted with an atom or a group selected from H, methyl, R_4 , hydroxyl, halogen, and amino, more preferably R_2 is substituted with an atom or a group selected from H, methyl, R_4 . More preferably R_2 is

individually selected from O, S, NH, N(Me), N(R₄), C(R₄)₂, CH(R₄) or CH₂, wherein R₄ is as defined below.

R₃ is methyl, beta-cyanoethyl, p-nitrophenetyl, o-chlorophenyl, or p-chlorophenyl.

5 R₄ is lower alkyl, preferably lower alkyl such as methyl, ethyl, or isopropyl, or heterocyclic, such as morpholino, pyrrolidino, or 2,2,6,6-tetramethylpyrrolidino, wherein lower alkyl is defined as C₁ - C₆, such as C₁ - C₄.

R₅ is alkyl, alkoxy, aryl or H, with the proviso that R₅ is H when X₂ = O⁻, preferably R₅ is selected from lower alkyl, lower alkoxy, aryloxy. In a preferred embodiment aryloxy is selected from phenyl, naphthyl or pyridine.

10 R₆ is a protecting group, selected from any suitable protecting groups. Preferably R₆ is selected from the group consisting of trityl, monomethoxytrityl, 2-chlorotrityl, 1,1,1,2-tetrachloro-2,2-bis(p-methoxyphenyl)-ethan (DATE), 9-phenylxanthine-9-yl (pixyl) and 9-(p-methoxyphenyl) xanthine-9-yl (MOX) or other protecting groups mentioned in "Current Protocols In Nucleic Acid Chemistry" volume 1, Beaucage et al. Wiley. More preferably, the
15 protecting group may be selected from the group consisting of monomethoxytrityl and dimethoxytrityl. Most preferably, the protecting group may be 4, 4'-dimethoxytrityl (DMT).

R₉ is selected from O, S, N optionally substituted, preferably R₉ is selected from O, S, NH, N(Me).

R₁₀ is selected from O, S, N, C, optionally substituted.

20 X₁ is selected from Cl, Br, I, or N(R₄)₂

X₂ is selected from Cl, Br, I, N(R₄)₂, or O⁻

As described above with respect to the substituents the backbone monomer unit can be acyclic or part of a ring system.

25 Preferably, the backbone monomer unit of an intercalator pseudonucleotide is selected from the group consisting of acyclic backbone monomer units. Acyclic is meant to cover any backbone monomer unit, which does not comprise a ring structure, for example the backbone monomer unit preferably does not comprise a ribose or a deoxyribose group.

30 In particular, it is preferred that the backbone monomer unit of an intercalator pseudonucleotide is an acyclic backbone monomer unit, which is capable of stabilising a bulge insertion (defined below).

The backbone monomer unit of an intercalator pseudonucleotide may be selected from the group consisting of backbone monomer units comprising at least one chemical group selected from trivalent and pentavalent phosphorous atom such as a pentavalent phosphorous atom. More preferably, the phosphate atom of the backbone monomer unit of an intercalator pseudonucleotide may be selected from the group consisting of backbone monomer units comprising at least one chemical group selected from the group consisting of, phosphoester, phosphodiester, phosphoramidate and phosphoramidite groups.

Preferred backbone monomer units comprising at least one chemical group selected from the group consisting of phosphate, phosphoester, phosphodiester, phosphoramidate and phosphoramidite groups are backbone monomer units, wherein the distance from at least one phosphor atom to at least one phosphor atom of a neighbouring nucleotide, not including the phosphor atoms, is at the most 6 atoms long, for example 2; such as 3, for example 4, such as 5, for example 6 atoms long, when the backbone monomer unit is incorporated into a nucleic acid backbone.

Preferably, the backbone monomer unit is capable of being incorporated into a phosphate backbone of a nucleic acid or nucleic acid analogue in a manner so that at the most 5 atoms (more preferably at most 4) are separating the phosphor atom of the intercalator pseudonucleotide backbone monomer unit and the nearest neighbouring phosphor atom, more preferably 5 atoms are separating the phosphor atom of the intercalator pseudonucleotide backbone monomer unit and the nearest neighbouring phosphor atom, in both cases not including the phosphor atoms themselves.

In a particularly preferred form, the intercalator pseudonucleotide comprises a backbone monomer unit that comprises a phosphoramidite and more preferably the backbone monomer unit comprises a trivalent phosphoramidite. Suitable trivalent phosphoramidites are trivalent phosphoramidites that may be incorporated into the backbone of a nucleic acid and/or a nucleic acid analogue. Usually, the amidit group may not be incorporated into the backbone of a nucleic acid, but rather the amidit group or part of the amidit group may serve as a leaving group and/ or protecting group. However, it is preferred that the backbone monomer unit comprises a phosphoramidite group because such a group may facilitate the incorporation of the backbone monomer unit into a nucleic acid backbone.

Preferably the acyclic backbone monomers may be selected from one of the general structures depicted in Figure 2.

Even more preferably, the backbone monomer unit includes optional protecting groups may be selected from the group consisting of the structures I) to XLIV) as indicated in Figure 2. Most preferred are the backbone monomer units selected structures I to VI.

Preferred acyclic backbone monomer unit may be selected from the group structures a) to g) in Figure 3.

The backbone monomer unit of an intercalator pseudonucleotide which is inserted into an oligonucleotide or oligonucleotide analogue, may comprise a phosphodiester bond. Additionally, the backbone monomer unit of an intercalator pseudonucleotide may comprise a pentavalent phosphoramidate. Preferably, the backbone monomer unit of an intercalator pseudonucleotide is an acyclic backbone monomer unit that may comprise a pentavalent phosphoramidate.

15 Leaving group

The backbone monomer unit may comprise one or more leaving groups. Leaving groups are chemical groups, which are part of the backbone monomer unit when the intercalator pseudonucleotide or the nucleotide is a monomer, but which are no longer present in the molecule once the intercalator pseudonucleotide or the nucleotide has been incorporated into an oligonucleotide or oligonucleotide analogue.

The nature of a leaving group depends of the backbone monomer unit. For example, when the backbone monomer unit is a phosphor amidit, the leaving group may, for example be an diisopropylamine group. In general, when the backbone monomer unit is a phosphor amidit, a leaving group is attached to the phosphor atom for example in the form of diisopropylamine and the leaving group is removed upon coupling of the phosphor atom to a nucleophilic group, whereas the rest of the phosphate group or part of the rest, may become part of the nucleic acid or nucleic acid analogue backbone.

Reactive group

The backbone monomer units may furthermore comprise a reactive group which is capable of performing a chemical reaction with another nucleotide or oligonucleotide or

nucleic acid or nucleic acid analogue to form a nucleic acid or nucleic acid analogue, which is one nucleotide longer than before the reaction. Accordingly, when nucleotides are in their free form, i.e. not incorporated into a nucleic acid, they may comprise a reactive group capable of reacting with another nucleotide or a nucleic acid or nucleic acid analogue.

5 The reactive group may be protected by a protecting group. Prior to the chemical reaction, the protection group may be removed. The protection group will thus not be a part of the newly formed nucleic acid or nucleic acid analogue. Examples of reactive groups are nucleophiles such as the 5'-hydroxy group of DNA or RNA backbone monomer units.

10 **Protecting group**

The backbone monomer unit may also comprise a protecting group which can be removed during synthesis. Removal of the protecting group allows for a chemical reaction between the intercalator pseudonucleotide and a nucleotide or nucleotide analogue or another intercalator pseudonucleotide.

15 In particular, a nucleotide monomer or nucleotide analogue monomer or intercalator pseudonucleotide monomer may comprise a protecting group, which is no longer present in the molecule once the nucleotide or nucleotide analogue or intercalator pseudonucleotide has been incorporated into a nucleic acid or nucleic acid analogue. Furthermore, backbone monomer units may comprise protecting groups which may be present in the oligonucleotide
20 or oligonucleotide analogue subsequent to incorporation of the nucleotide or nucleotide analogue or intercalator pseudonucleotide, but which may no longer be present after introduction of an additional nucleotide or nucleotide analogue to the oligonucleotide or oligonucleotide analogue or which may be removed after the synthesis of the entire oligonucleotide or oligonucleotide analogue.

25 The protecting group may be removed by a number of suitable techniques known to the person skilled in the art. Preferably, the protecting group may be removed by a treatment selected from the group consisting of acid treatment, thiophenol treatment and alkali treatment.

30 Preferred protecting groups, which may be used to protect the 5' end or the 5' end analogue of a backbone monomer unit may be selected from the group consisting of trityl, monomethoxytrityl, 2-chlorotrityl, 1,1,1,2-tetrachloro-2,2-bis(p-methoxyphenyl)-ethan

(DATE), 9-phenylxanthine-9-yl (pixyl) and 9-(p-methoxyphenyl) xanthine-9-yl (MOX) or other protecting groups mentioned in "Current Protocols In Nucleic Acid Chemistry" volume 1, Beaucage et al. Wiley. More preferably the protecting group may be selected from the group consisting of monomethoxytrityl and dimethoxytrityl. Most preferably, the protecting group may be 4, 4'-dimethoxytrityl(DMT). 4, 4'-dimethoxytrityl(DMT) groups may be removed by acid treatment, for example by brief incubation (30 to 60 seconds sufficient) in 3% trichloroacetic acid or in 3% dichloroacetic acid in CH_2Cl_2 .

Preferred protecting groups which may protect a phosphate or phosphoramidite group of a backbone monomer unit may for example be selected from the group consisting of methyl and 2-cyanoethyl. Methyl protecting groups may for example be removed by treatment with thiophenol or disodium 2-carbamoyl 2-cyanoethylene- 1,1-dithiolate. 2-cyanoethyl-groups may be removed by alkali treatment, for example treatment with concentrated aqueous ammonia, a 1:1 mixture of aqueous methylamine and concentrated aqueous ammonia or with ammonia gas.

Intercalator

The term intercalator covers any molecular moiety comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid. Preferably an intercalator consists of at least one essentially flat conjugated system which is capable of co-stacking with nucleobases of a nucleic acid or nucleic acid analogue.

Preferably, the intercalator comprises a chemical group selected from the group consisting of polyaromates and heteropolyaromates an even more preferably the intercalator essentially consists of a polyaromate or a heteropolyaromate. Most preferably, the intercalator is selected from the group consisting of polyaromates and heteropolyaromates.

Polyaromates or heteropolyaromates may consist of any suitable number of rings, such as 1, for example 2, such as 3, for example 4, such as 5, for example 6, such as 7, for example 8, such as more than 8. Furthermore polyaromates or heteropolyaromates may be substituted with one or more selected from the group consisting of hydroxyl, bromo, fluoro, chloro, iodo, mercapto, thio, cyano, alkylthio, heterocycle, aryl, heteroaryl, carboxyl, carboalkoyl, alkyl, alkenyl, alkynyl, nitro, amino, alkoxy and amido.

In one preferred form, the intercalator may be selected from the group consisting of polyaromates and heteropolyaromates that are capable of fluorescing.

In another more preferred form, the intercalator may be selected from the group consisting of polyaromates and heteropolyaromates that are capable of forming excimers, exciplexes, fluorescence resonance energy transfer (FRET) or charged transfer complexes.

Accordingly, the intercalator may preferably be selected from the group consisting of phenanthroline, phenazine, phenanthridine, anthraquinone, pyrene, anthracene, naphthene, phenanthrene, picene, chrysene, naphhtacene, acridones, benzanthracenes, stilbenes, oxalo-pyridocarbazoles, azidobenzenes, porphyrins, psoralens and any of the aforementioned intercalators substituted with one or more selected from the group consisting of hydroxyl, bromo, fluoro, chloro, iodo, mercapto, thio, cyano, alkylthio, heterocycle, aryl, heteroaryl, carboxyl, carboalkoyl, alkyl, alkenyl, alkynyl, nitro, amino, alkoxyl and/or amido.

Preferably, the intercalator is selected from the group consisting of phenanthroline, phenazine, phenanthridine, anthraquinone, pyrene, anthracene, naphthene, phenanthrene, picene, chrysene, naphhtacene, acridones, benzanthracenes, stilbenes, oxalo-pyridocarbazoles, azidobenzenes, porphyrins and psoralens.

More preferably the intercalator may be selected from the group of intercalators comprising one of the structures as indicated in Figure 4 as well as derivatives thereof:

Even more preferably the intercalator may be selected from the group of intercalators comprising one of the intercalator structures above numbered V, XII, XIV, XV, XVII, XXIII, XXVI, XXVIII, XLVII, LI and LII of Figure 4 or derivatives thereof.

Most preferably, the intercalator is selected from the group of intercalator structures above numbered XII, XIV, XVII, XXIII, LI of Figure 4.

The examples of intercalators of Figure 4 are not to be understood as limiting in any way, but only as to provide examples of possible structures for use as intercalators. In addition, the substitution of one or more chemical groups on each intercalator to obtain modified structures is also included.

The intercalator moiety of the intercalator pseudonucleotide is linked to the backbone unit by the linker. When going from the backbone along the linker to the intercalating moiety, the linker and intercalator connection is defined as the bond between a linker atom

and the first atom being part of a conjugated system that is able to co-stack with nucleobases of a strand of a oligonucleotide or oligonucleotide analogue when the oligonucleotide or oligonucleotide analogue is hybridized to an oligonucleotide analogue comprising the intercalator pseudonucleotide.

- 5 The linker may comprise a conjugated system and the intercalator may comprise another conjugated system. In this case the linker conjugated system is not capable of co-stacking with nucleobases of the opposite oligonucleotide or oligonucleotide analogue strand.

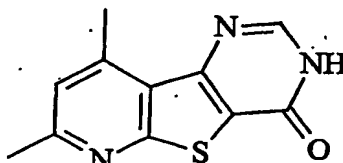
10 **Linker**

The linker of a intercalator pseudonucleotide is a moiety connecting the intercalator and the backbone monomer of the intercalator pseudonucleotide. The linker may comprise one or more atom(s) or bond(s) between atoms.

- 15 By the definitions of backbone and intercalating moieties defined herein, the linker is the shortest path linking the backbone and the intercalator. If the intercalator is linked directly to the backbone, the linker is a bond. The linker usually consists of a chain of atoms or a branched chain of atoms. Chains can be saturated as well as unsaturated. The linker may also be a ring structure with or without conjugated bonds. For example, the linker may comprise a chain of m atoms selected from the group consisting of C, O, S, N, P, Se, Si, Ge, 20 Sn and Pb, wherein one end of the chain is connected to the intercalator and the other end of the chain is connected to the backbone monomer unit.

- 25 The total length of the linker and the intercalator of the intercalator pseudonucleotides preferably is between 8 and 13 Å. Accordingly, m should be selected dependent on the size of the intercalator of the specific intercalator pseudonucleotide. That is, m should be relatively large, when the intercalator is small and m should be relatively small when the intercalator is large. For most purposes, however, m will be an integer from 1 to 7, such as from 1 to 6, such as from 1 to 5, such as from 1 to 4. As described above, the linker may be an unsaturated chain or another system involving conjugated bonds. For example, the linker may comprise cyclic conjugated structures. Preferably, m is from 1 to 4 30 when the linker is an saturated chain.

When the intercalator is pyrene, m is preferably an integer from 1 to 7, such as from 1 to 6, such as from 1 to 5, such as from 1 to 4, more preferably from 1 to 4, even more preferably from 1 to 3, most preferably m is 2 or 3.



When the intercalator has the structure
 m is preferably from 2 to 6, more preferably 2.

The chain of the linker may be substituted with one or more atoms selected from the group consisting of C, H, O, S, N, P, Se, Si, Ge, Sn and Pb.

In one form, the linker is an azaalkyl, oxaalkyl, thiaalkyl or alkyl chain. For example, the linker may be an alkyl chain substituted with one or more selected from the group consisting of C, H, O, S, N, P, Se, Si, Ge, Sn and Pb. In a preferred embodiment the linker consists of an unbranched alkyl chain, wherein one end of the chain is connected to the intercalator and the other end of the chain is connected to the backbone monomer unit and wherein each C is substituted with 2 H.. More preferably, the unbranched alkyl chain is from 1 to 5 atoms long, such as from 1 to 4 atoms long, such as from 1 to 3 atoms long, such as from 2 to 3 atoms long.

In another form, the linker is a ring structure comprising atoms selected from the group consisting of C, O, S, N, P, Se, Si, Ge, Sn and Pb. For example the linker may be such a ring structure substituted with one or more selected from the group consisting of C, H, O, S, N, P, Se, Si, Ge, Sn and Pb.

In another form, the linker consists of from 1 to 6 C atoms, from 0 to 3 of each of the following atoms O, S, N. More preferably the linker consists of from 1 to 6 C atoms and from 0 to 1 of each of the atoms O, S, N. In a preferred form, the linker consists of a chain of C, O, S and N atoms, optionally substituted. Preferably the chain should consist of at the most 3 atoms, thus comprising from 0 to 3 atoms selected individually from C, O, S, N, optionally substituted.

In a preferred form, the linker consists of a chain of C, N, S and O atoms, wherein one end of the chain is connected to the intercalator and the other end of the chain is

connected to the backbone monomer unit. Preferably such a chain comprises one of the linkers shown in Figure 5.

In a more preferred form, the chain comprises one of the linkers II, III, VI, or IX shown in Figure 5.

- 5 The linker constitutes Y in the formula for the intercalator pseudonucleotide X-Y-Q, as defined above, and hence X and Q are not part of the linker.

Intercalator pseudonucleotides

- 10 Intercalator pseudonucleotides or INA molecules preferably have the general structure



wherein

- 15 X is a backbone monomer unit capable of being incorporated into the backbone of a nucleic acid or nucleic acid analogue;

Q is an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid; and

Y is a linker moiety linking the backbone monomer unit and the intercalator;

- 20 wherein the total length of Q and Y is in the range from about 7 Å to 20 Å.

Furthermore, in a preferred embodiment of the present invention the intercalator pseudonucleotide comprises a backbone monomer unit, wherein the backbone monomer unit is capable of being incorporated into the phosphate backbone of a nucleic acid or nucleic acid analogue in a manner so that at the most 4 atoms are separating the two phosphor atoms of the backbone that are closest to the intercalator.

25

The intercalator pseudonucleotides preferably do not comprise a nucleobase capable of forming Watson-Crick hydrogen bonding. Hence intercalator pseudonucleotides are preferably not capable of Watson-Crick base pairing.

Preferably, the total length of Q and Y is in the range from about 7 Å to 20 Å, more preferably, from about 8 Å to 15 Å, even more preferably from about 8 Å to 13 Å, even more preferably from about 8.4 Å to 12 Å, most preferably from about 8.59 Å to 10 Å or from about 8.4 Å to 10.5 Å.

5. When the intercalator is pyrene for example, the total length of Q and Y is preferably in the range of about 8 Å to 13 Å, such as from about 9 Å to 13 Å, more preferably from about 9.05 Å to 11 Å, such as from about 9.0 Å to 11 Å, even more preferably from about 9.05 to 10 Å, such as from about 9.0 to 10 Å, most preferably about 9.8 Å.

10 The total length of the linker (Y) and the intercalator (Q) should be determined by determining the distance from the center of the non-hydrogen atom of the linker which is furthest away from the intercalator to the center of the non-hydrogen atom of the essentially flat, conjugated system of the intercalator that is furthest away from the backbone monomer unit. Preferably, the distance should be the maximal distance in which bonding angles and normal chemical laws are not broken or distorted in any way.

15 The distance should preferably be determined by calculating the structure of the free intercalating pseudonucleotide with the lowest conformational energy level, and then determining the maximum distance that is possible from the center of the non-hydrogen atom of the linker which is furthest away from the intercalator to the center of the non-hydrogen atom of the essentially flat, conjugated system of the intercalator that is furthest
20 away from the backbone monomer unit without bending, stretching or otherwise distorting the structure more than simple rotation of bonds that are free to rotate (e.g. not double bonds or bonds participating in a ring structure). Preferably the energetically favorable structure is found by *ab initio* or force fields calculations.

The distance can be determined by a method consisting of the following steps:
25 the structure of the intercalator pseudonucleotide of interest is drawn by computer using the programme ChemWindow® 6.0 (BioRad);
the structure is transferred to the computer programme SymApps™ (BioRad);
the 3-dimensional structure comprising calculated lengths of bonds and bonding angles of the intercalator pseudonucleotide is calculated using the computer programme
30 SymApps™ (BioRad);

the 3 dimensional structure is transferred to the computer programme RasWin Molecular Graphics Ver. 2.6-ucb;

the bonds are rotated using RasWin Molecular Graphics Ver. 2.6-ucb to obtain the maximal distance (the distance as defined herein above); and

5 the distance is determined.

Intercalator pseudonucleotides may be any combination of the above mentioned backbone monomer units, linkers and intercalators.

Examples of intercalator pseudonucleotides are shown in Figure 6. In a preferred form, the intercalator pseudonucleotide is selected structures 1 to 9 shown in Figure 6.

10 In another preferred form, the intercalator pseudonucleotide is selected from the group consisting of phosphoramidites of 1-(4,4'-dimethoxytriphenylmethyloxy)-3-pyrenemethyloxy-2-propanol. Even more preferably, the intercalator pseudonucleotide is selected from the group consisting of the phosphoramidite of (S)-1-(4,4'-dimethoxytriphenylmethyloxy)-3-pyrenemethyloxy-2-propanol and the phosphoramidite of
15 (R)-1-(4,4'-dimethoxytriphenylmethyloxy)-3-pyrenemethyloxy-2-propanol.

Preparation of intercalator pseudonucleotides

The intercalator pseudonucleotides or INA molecules may be synthesised by any suitable method. One suitable method comprises the steps of

- 20 a1) providing a compound containing an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid and optionally a linker part coupled to a reactive group;
- b1) providing a linker precursor molecule comprising at least two reactive groups, the two reactive groups may optionally be individually protected; and
- 25 c1) reacting the intercalator with the linker precursor and thereby obtaining an intercalator-linker;
- d1) providing a backbone monomer precursor unit comprising at least two reactive groups, the two reactive groups may optionally be individually protected and/or masked and optionally comprising a linker part; and

- e1) reacting the intercalator-linker with the backbone monomer precursor and obtaining an intercalator-linker-backbone monomer precursor;
- or
- a2) providing a backbone monomer precursor unit comprising at least two reactive groups, the two reactive groups may optionally be individually protected and/or masked and optionally comprising a linker part;
- 5 b2) providing a linker precursor molecule comprising at least two reactive groups, the two reactive groups may optionally be individually protected;
- c2) reacting the monomer precursor unit with the linker precursor and thereby obtaining a backbone-linker;
- 10 d2) providing a compound containing an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid and optionally a linker part coupled to a reactive group; and
- e2) reacting the intercalator with the backbone-linker and obtaining an intercalator-linker-backbone monomer precursor;
- 15 or
- a3) providing a compound containing an intercalator comprising at least one essentially flat conjugated system, which is capable of co-stacking with nucleobases of a nucleic acid and a linker part coupled to a reactive group;
- 20 b3) providing a backbone monomer precursor unit comprising at least two reactive groups, the two reactive groups may optionally be individually protected and/or masked), and a linker part;
- c3) reacting the intercalator-linker part with the backbone monomer precursor-linker and obtaining an intercalator-linker-backbone monomer precursor;
- 25 f) optionally protecting and/ or de-protecting the intercalator-linker-backbone monomer precursor;
- g) providing a phosphor containing compound capable of linking two psuedonucleotides, nucleotides and/ or nucleotide analogues together;
- h) reacting the phosphorous containing compound with the intercalator-linker-backbone monomer precursor; and
- 30

- i) obtaining an intercalator pseudonucleotide.

Preferably, the intercalator reactive group is selected so that it may react with the linker reactive group. Hence, if the linker reactive group is a nucleophile, then preferably the intercalator reactive group is an electrophile, more preferably an electrophile selected from the group consisting of halo alkyl, mesyloxy alkyl and tosyloxy alkyl. More preferably the intercalator reactive group is chloromethyl. Alternatively, the intercalator reactive group may be a nucleophile group for example a nucleophile group comprising hydroxy, thiol, selam, amine or mixture thereof.

Preferably, the cyclic or non cyclic alkane may be a poly-substituted alkane or alkoxy comprising at least three linker reactive groups. More preferably the poly-substituted alkane may comprise three nucleophilic groups such as, but not limited to, an alkane triole, an aminoalkane diol or mercaptoalkane diol. Preferably the poly-substituted alkane contain one nucleophilic group that is more reactive than the others, alternatively two of the nucleophilic groups may be protected by a protecting group. More preferably the cyclic or non cyclic alkane is 2,2-dimethyl-4-methylhydroxy-1,3-dioxalan, even more preferably the alkane is D- α,β -isopropylidene glycerol.

Preferably, the linker reactive groups should be able to react with the intercalator reactive groups, for example the linker reactive groups may be a nucleophile group for example selected from the group consisting of hydroxy, thiol, selam and amine, preferably a hydroxy group. Alternatively the linker reactive group may be an electrophile group, for example selected from the group consisting of halogen, triflates, mesylates and tosylates. In a preferred form, at least 2 linker reactive groups may be protected by a protecting group.

The method may further comprise a step of attaching a protecting group to one or more reactive groups of the intercalator-precursor monomer. For example a DMT group may be added by providing a DMT coupled to a halogen, such as Cl, and reacting the DMT-Cl with at least one linker reactive group. Accordingly, preferably at least one linker reactive group will be available and one protected. If this step is done prior to reaction with the phosphor comprising agent, then the phosphor comprising agent may only interact with one linker reactive group.

The phosphor comprising agent may for example be a phosphoramidite, for example $\text{NC}(\text{CH}_2)_2\text{OP}(\text{Npr}^1)_2$ or $\text{NC}(\text{CH}_2)_2\text{OP}(\text{Npr}^1)_2\text{Cl}$. Preferably the phosphor comprising agent may

be reacted with the intercalator-precursor in the presence of a base, such as $N(et)_3$, $N('pr)_2Et$ and CH_2Cl_2 .

One specific example of a method of synthesising an intercalator pseudonucleotide is outlined in example 1 and in Figure 7.

5 Once the appropriate sequences of oligonucleotide or oligonucleotide analogue are determined, they are preferably chemically synthesised using commercially available methods and equipment. For example, the solid phase phosphoramidite method can be used to produce short oligonucleotide or oligonucleotide analogue comprising intercalator pseudonucleotides.

10 For example the oligonucleotides or oligonucleotide analogues may be synthesised by any of the methods described in "Current Protocols in Nucleic acid Chemistry" Volume 1, Beaucage et al., Wiley.

Oligonucleotides comprising intercalator pseudonucleotides

15 High affinity of synthetic nucleic acids towards target nucleic acids may greatly facilitate detection assays and furthermore synthetic nucleic acids with high affinity towards target nucleic acids may be useful for a number of other purposes, such as gene targeting and purification of nucleic acids. Oligonucleotides or oligonucleotide analogues comprising intercalators have been shown to increase affinity for homologous complementary nucleic acids.

20 Oligonucleotides or oligonucleotide analogues comprising at least one intercalator pseudonucleotide can be made wherein the melting temperature of a hybrid consisting of the oligonucleotides or oligonucleotide analogues and a homologous complementary DNA (DNA hybrid) is significantly higher than the melting temperature of a hybrid between an oligonucleotide or oligonucleotide analogue lacking intercalator pseudonucleotide(s) consisting of the same nucleotide sequence as the oligonucleotide or oligonucleotide analogue and the homologous complementary DNA (corresponding DNA hybrid).

25 Preferably, the melting temperature of the DNA hybrid is from 1 to 80°C, more preferably at least 2°C, even more preferably at least 5°C, yet more preferably at least 10°C higher than the melting temperature of the corresponding DNA hybrid.

30

Oligonucleotides or oligonucleotide analogues can have at least one internal intercalator pseudonucleotide. Positioning intercalator units internally allows for greater flexibility in design. Nucleic acid analogues comprising internally positioned intercalator pseudonucleotides may thus have higher affinity for homologous complementary nucleic acids than nucleic acid analogues that does not have internally positioned intercalator pseudonucleotides. Oligonucleotides or Oligonucleotide analogues comprising at least one internal intercalator pseudonucleotide may also be able to discriminate between RNA (including RNA-like nucleic acid analogues) and DNA (including DNA-like nucleic acid analogues). Furthermore internally positioned fluorescent intercalator monomers could find use in diagnostic tools.

The intercalator pseudonucleotides may be placed in any desirable position within a given oligonucleotide or oligonucleotide analogue. For example, an intercalator pseudonucleotide may be placed at the end of the oligonucleotide or oligonucleotide analogue or an intercalator pseudonucleotide may be placed in an internal position within the oligonucleotide or oligonucleotide analogue.

When the oligonucleotide or oligonucleotide analogue comprise more than 1 intercalator pseudonucleotide, the intercalator pseudonucleotides may be placed in any position in relation to each other. For example they may be placed next to each other, or they may be positioned so that 1, such as 2, for example 3, such as 4, for example 5, such as more than 5 nucleotides are separating the intercalator pseudonucleotides. In one preferred embodiment two intercalator pseudonucleotides within an oligonucleotide or oligonucleotide analogue are placed as next nearest neighbours, i.e. they can be placed at any position within the oligonucleotide or oligonucleotide analogue and having 1 nucleotide separating the two intercalator pseudonucleotides. In another preferred form, two intercalators are placed at or in close proximity to each end respectively of the oligonucleotide or oligonucleotide analogue.

The oligonucleotides or oligonucleotide analogues may comprise any kind of nucleotides and/or nucleotide analogues, such as the nucleotides and/or nucleotide analogues described herein above. For example, the oligonucleotides or oligonucleotide analogues may comprise nucleotides and/or nucleotide analogues comprised within DNA, RNA, LNA, PNA, ANA and HNA. Accordingly, the oligonucleotides or oligonucleotide analogue may comprise one or more selected from the group consisting of subunits of PNA,

Homo-DNA, b-D-Altropyranosyl-NA, b-D-Glucopyranosyl-NA, b-D-Allopyranosyl-NA, HNA, MNA, ANA, LNA, CNA, CeNA, TNA, (2'-NH)-TNA, (3'-NH)-TNA, \square -L-Ribo-LNA, \square -L-Xylo-LNA, \square -D-Xylo-LNA, \square -D-Ribo-LNA, [3.2.1]-LNA, Bicyclo-DNA, 6-Amino-Bicyclo-DNA, 5-epi-Bicyclo-DNA, \square -Bicyclo-DNA, Tricyclo-DNA, Bicyclo[4.3.0]-DNA, Bicyclo[3.2.1]-DNA, Bicyclo[4.3.0]amide-DNA, \square -D-Ribopyranosyl-NA, \square -L-Lyxopyranosyl-NA, 2'-R-RNA, 2'-OR-RNA, \square -L-RNA, α -D-RNA, β -D-RNA, i.e. the oligonucleotide analogue may be selected from the group of PNA, Homo-DNA, b-D-Altropyranosyl-NA, b-D-Glucopyranosyl-NA, b-D-Allopyranosyl-NA, HNA, MNA, ANA, LNA, CNA, CeNA, TNA, (2'-NH)-TNA, (3'-NH)-TNA, \square -L-Ribo-LNA, \square -L-Xylo-LNA, \square -D-Xylo-LNA, \square -D-Ribo-LNA, [3.2.1]-LNA, Bicyclo-DNA, 6-Amino-Bicyclo-DNA, 5-epi-Bicyclo-DNA, \square -Bicyclo-DNA, Tricyclo-DNA, Bicyclo[4.3.0]-DNA, Bicyclo[3.2.1]-DNA, Bicyclo[4.3.0]amide-DNA, \square -D-Ribopyranosyl-NA, \square -L-Lyxopyranosyl-NA, 2'-R-RNA, 2'-OR-RNA, \square -L-RNA, α -D-RNA, β -D-RNA and mixtures thereof.

One advantage of the oligonucleotides or oligonucleotide analogues is that the melting temperature of a hybrid consisting of an oligonucleotide or oligonucleotide analogue comprising at least one intercalator pseudonucleotide and an essentially complementary DNA (DNA hybrid) is significantly higher than the melting temperature of a duplex consisting of the essentially complementary DNA and a DNA complementary thereto.

Accordingly, oligonucleotides or oligonucleotide analogues may form hybrids with DNA with higher affinity than naturally occurring nucleic acids. The melting temperature is preferably increased with 2 to 30°C, for example from 5 to 20°C, such as from 10°C to 15°C, for example from 2°C to 5°C, such as from 5°C to 10°C, such as from 15°C to 20°C, for example from 20°C to 25°C, such as from 25°C to 30°C, for example from 30°C to 35°C, such as from 35°C to 40°C, for example from 40°C to 45°C, such as from 45°C to 50°C higher.

In particular, the increase in melting temperature may be achieved due to intercalation of the intercalator, because the intercalation may stabilise a DNA duplex. Accordingly, it is preferred that the intercalator is capable of intercalating between nucleobases of DNA. Preferably, the intercalator pseudonucleotides are placed as bulge insertions or end insertions in the duplex (see below), which in some nucleic acids or nucleic acid analogues may allow for intercalation.

The melting temperature of an oligonucleotide or oligonucleotide analogue comprising at least one intercalator pseudonucleotide and an essentially complementary

RNA (RNA hybrid) or a RNA-like nucleic acid analogue (RNA-like hybrid) can be significantly higher than the melting temperature of a duplex consisting of the essentially complementary RNA or RNA-like target and the oligonucleotide analogue comprising no intercalator pseudonucleotides. Preferably most or all of the intercalator pseudonucleotides of the oligonucleotide or oligonucleotide analogue are positioned at either or both ends.

Accordingly, oligonucleotides and/or oligonucleotide analogues may form hybrids with RNA or RNA-like nucleic acid analogues or RNA-like oligonucleotide analogues with higher affinity than naturally occurring nucleic acids. The melting temperature is preferably increased with from 2 to 20°C, for example from 5 to 15°C, such as from 10°C to 15°C, for example from 2°C to 5°C, such as from 5°C to 10°C, such as from 15°C to 20°C or higher.

The intercalator pseudonucleotides will preferably only stabilise towards RNA and RNA-like targets when positioned at the end of the oligonucleotide or oligonucleotide analogue. This does not however exclude the positioning of intercalator pseudonucleotides in oligonucleotides or oligonucleotide analogues to be hybridized with RNA or RNA-like nucleic acid analogues such that the intercalator pseudonucleotides are placed in regions internal to the formed hybrid. This may be done to obtain certain hybrid instabilities or to affect the overall 2D or 3D structure of both intra- and inter-molecular complexes to be formed subsequent to hybridisation.

An oligonucleotide and/or oligonucleotide analogue comprising one or more intercalator pseudonucleotides may form a triple stranded structure (triplex-structure) consisting of the oligonucleotide and/or oligonucleotide analogue bound by Hoogsteen base pairing to a homologous complementary nucleic acid or nucleic acid analogue or oligonucleotide or oligonucleotide analogue. The oligonucleotide or oligonucleotide analogue may increase the melting temperature of the Hoogsteen base pairing in the triplex-structure.

The oligonucleotide or oligonucleotide analogue may increase the melting temperature of the Hoogsteen base pairing in the triplex-structure in a manner not dependent on the presence of specific sequence restraints like purine-rich / pyrimidine-rich nucleic acid or nucleic acid analogue duplex target sequences. Accordingly, the Hoogsteen base pairing in the triplex-structure has significantly higher melting temperature than the melting temperature of the Hoogsteen base pairing to the duplex target if the oligonucleotide or oligonucleotide analogue had no intercalator pseudonucleotides.

Accordingly, oligonucleotides or oligonucleotide analogues may form triplex-structures with homologous complementary nucleic acid or nucleic acid analogue or oligonucleotide or oligonucleotide analogue with higher affinity than naturally occurring nucleic acids. The melting temperature is preferably increased with from 2 to 50°C, such as from 2 to 40°C, such as from 2 to 30°C, for example from 5 to 20°C, such as from 10°C to 15°C, for example from 2°C to 5°C, such as from 5°C to 10°C, for example from 10°C to 15°C, such as from 15°C to 20°C, for example from 20°C to 25°C, such as from 25°C to 30°C, for example from 30°C to 35°C, such as from 35°C to 40°C, for example from 40°C to 45°C, such as from 45°C to 50°C.

10 In particular, the increase in melting temperature may be achieved due to intercalation of the intercalator, because the intercalation may stabilise a DNA triplex. Accordingly, it is preferred that the intercalator is capable of intercalating between nucleobases of a triplex-structure. Preferably, the intercalator pseudonucleotide is placed as a bulge insertion in the duplex (see below); which in some nucleic acids or nucleic acid
15 analogues may allow for intercalation.

Triplex-formation may or may not proceed in strand invasion, a process where the Hoogsteen base-paired third strand invades the target duplex and displaces part or all of the identical strand to form Watson-Crick base pairs with the complementary strand. This can be exploited for several purposes. The oligonucleotides and oligonucleotides are suitably used
20 if only double stranded nucleic acid or nucleic acid analogue target is present and it is not possible, feasible or wanted to separate the target strands, detection by single strand invasion of the region or double strand invasion of complementary regions, without prior melting of double stranded nucleic acid or nucleic acid analogue target, for triplex-formation and/or strand invasion. Accordingly, an oligonucleotide or oligonucleotide analogue
25 comprising at least one intercalator pseudonucleotide is provided that is able to invade a double stranded region of a nucleic acid or nucleic acid analogue molecule.

An oligonucleotide or oligonucleotide analogue comprising at least one intercalator pseudonucleotide that is able to invade a double stranded nucleic acid or nucleic acid
analogue in a sequence specific manner can be provided. Invading oligonucleotide and/or
30 oligonucleotide analogue comprising at least one intercalator pseudonucleotide will bind to the complementary strand in a sequence specific manner with higher affinity than the strand displaced.

The melting temperature of a hybrid consisting of an oligonucleotide analogue comprising at least one intercalator pseudonucleotide and a homologous complementary DNA (DNA hybrid), is usually significantly higher than the melting temperature of a hybrid consisting of the oligonucleotide or oligonucleotide analogue and a homologous complementary RNA (RNA hybrid) or RNA-like nucleic acid analogue target or RNA-like oligonucleotide analogue target. The oligonucleotide may be any of the above described oligonucleotide analogues. For example, the oligonucleotide may be a DNA oligonucleotide (analogue) comprising at least one intercalator pseudonucleotide or a Homo-DNA, b-D-Altropyranosyl-NA, b-D-Glucopyranosyl-NA, b-D-Allopyranosyl-NA, HNA, MNA, ANA, LNA, CNA, CeNA, TNA, (2'-NH)-TNA, (3'-NH)-TNA, \square -L-Ribo-LNA, \square -L-Xylo-LNA, \square -D-Xylo-LNA, \square -D-Ribo-LNA, [3.2.1]-LNA, Bicyclo-DNA, 6-Amino-Bicyclo-DNA, 5-epi-Bicyclo-DNA, \square -Bicyclo-DNA, Tricyclo-DNA, Bicyclo[4.3.0]-DNA, Bicyclo[3.2.1]-DNA, Bicyclo[4.3.0]amide-DNA, \square -D-Ribopyranosyl-NA, \square -L-Lyxopyranosyl-NA, 2'-R-RNA, 2'-OR-RNA, \square -L-RNA, α -D-RNA, β -D-RNA oligonucleotide or mixtures hereof comprising at least one intercalator pseudonucleotide.

Accordingly, the affinity of the oligonucleotide or oligonucleotide analogue for DNA is significantly higher than the affinity of the oligonucleotide or oligonucleotide analogue for RNA or an RNA-like target. Hence in a mixture comprising a limiting number of the oligonucleotide or oligonucleotide analogue and a homologous complementary DNA and a homologous complementary RNA or homologous complementary RNA-like target, the oligonucleotide or oligonucleotide analogue will preferably hybridize to the homologous complementary DNA.

Preferably, the melting temperature of the DNA hybrid is at least 2°C, such as at least 5°C, for example at least 10°C, such as at least 15°C, for example at least 20°C, such as at least 25°C, for example at least 30°C, such as at least 35°C, for example at least 40°C, such as from 2 to 30°C, for example from 5 to 20°C, such as from 10°C to 15°C, for example from 2°C to 5°C, such as from 5°C to 10°C, for example from 10°C to 15°C, such as from 15°C to 20°C, for example from 20°C to 25°C, such as from 25°C to 30°C, for example from 30°C to 35°C, such as from 35°C to 40°C, for example from 40°C to 45°C, such as from 45°C to 50°C, for example from 50°C to 55°C, such as from 55°C to 60°C higher than the melting temperature of a homologous complementary RNA or RNA-like hybrid.

An oligonucleotide or oligonucleotide analogue containing at least one intercalator pseudonucleotide can be hybridized to secondary structures of nucleic acids or nucleic acid analogues. The oligonucleotide or oligonucleotide analogue is capable of stabilizing such a hybridization to the secondary structure. Secondary structures could be, but are not limited to, stem-loop structures, Faraday junctions, fold-backs, H-knots, and bulges. The secondary structure can be a stem-loop structure of RNA, where an oligonucleotide or oligonucleotide analogue comprising at least one intercalator pseudonucleotide is designed in a way so the intercalator pseudonucleotide is hybridizing at the end of one of the three duplexes formed in the three-way junction between the secondary structure and the oligonucleotide or oligonucleotide analogue.

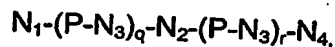
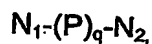
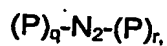
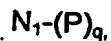
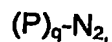
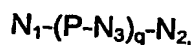
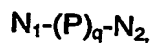
Position of intercalator pseudonucleotide

An oligonucleotide or oligonucleotide analogue can be designed in a manner so it may hybridize to a homologous complementary nucleic acid or nucleic acid analogue (target nucleic acid). Preferably, the oligonucleotide or oligonucleotide analogue may be substantially complementary to the target nucleic acid. More preferably, at least one intercalator pseudonucleotide is positioned so that when the oligonucleotide analogue is hybridized with the target nucleic acid, the intercalator pseudonucleotide is positioned as a bulge insertion, i.e. the upstream neighbouring nucleotide of the intercalator pseudonucleotide and the downstream neighbouring nucleotide of the intercalator pseudonucleotide are hybridized to neighbouring nucleotides in the target nucleic acid.

An intercalator pseudonucleotide can be positioned next to either or both ends of a duplex formed between the oligonucleotide analogue comprising the intercalator pseudonucleotide and its target nucleotide or nucleotide analogue, for example the intercalator pseudonucleotide may be positioned as a dangling, co-stacking end.

All intercalator pseudonucleotides or INA of an oligonucleotide or oligonucleotide analogue can be positioned so that when the oligonucleotide analogue is hybridized with the target nucleic acid, all intercalator pseudonucleotides are positioned as bulge insertions and/or as dangling, co-stacking ends.

Examples of oligonucleotides containing intercalator pseudonucleotides are depicted below:



5

10 wherein

N_1 , N_2 , N_3 , N_4 individually denotes a sequence of nucleotides and/or nucleotides analogues of at least one nucleotide,

P denotes an intercalator pseudonucleotide, and

q and r are individually selected from an integer of from 1 to 10.

15

EXAMPLES

Example 1

Preparation of an intercalator pseudonucleotide or INA

1-Pyrenemethanol is commercially available, but it is also easily prepared from
20 pyrene by Vilsmeier-Haack formulation followed by reduction with sodium borohydride and subsequent conversion of the alcohol with thionyl chloride affords 1-(chloromethyl)pyrene in 98% yield.

The acyclic amidite **5** (Figure 7) was prepared from (S)-(+)-2,2-dimethyl-1,3-dioxalane-4-methanol and 1-(chloromethyl)pyrene in 52% overall yield. The synthesis of **5**
25 (Figure 7) is accomplished using KOH for the alkylation reaction, and using 80% aqueous acetic acid to give the diol **3** (Figure 7), which is protected with dimethoxytritylchloride (DMT-Cl) and finally reaction with 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite affords target compound **5** (Figure 7) in 72% yield. The yield in the latter reaction step was

decreased from 72% to 14% when 2-cyanoethyl *N,N*-diisopropylchlorophosphor amidite was used as the phosphorylating reagent. The synthesis of the acyclic amidite 5 is shown schematically in Figure 7.

5 *1-Pyrenylcarbaldehyde*

A mixture of *N*-formyl-*N*-methylaniline (68.0 g; 41.4 ml; 503 mmol) and *o*-dichlorobenzene (75 mL) was cooled on an ice bath and added phosphoroxchloride (68g; 440 mmol) over 2 hours so that the temperature do not exceed 25°C. Pulverized Pyrene (50 g; 247 mmol) was added in small portions over 30 min and the reaction mixture is equipped with a condenser and heated at 90-95°C for 2 hours. After cooling to room temperature the dark red compound was filtered off and washed with benzene (50 ml). Then it was transferred to water (250 ml) and stirred over night. The yellow aldehyde was filtered and washed with water (3×50 ml). Recrystallized from 75% ethanol 3 times. Yield: 30.0 g (52.7%).

15 *1-Pyrenylmethanol*

1-Pyrenylcarbaldehyde (10.0 g; 43.4 mmol) was dissolved in dry THF (50 ml) under inert atmosphere and NaBH₄ (0.82 g; 22 mmol) was added in small portions over 10 min. The reaction mixture was stirred over night at room temperature and crystallizing the product by pouring into stirring water (350 ml). The product is filtered, washed with water (4×25 ml) and dried under reduced pressure. Recrystallized from ethyl acetate. Yield: 8.54 g (84.7%).

1-(Chloromethyl)-pyrene

1-Pyrenylmethanol (6.40 g; 27.6 mmol) was dissolved in a mixture of pyridine (3.3 ml; 41.3 mmol) and CH₂Cl₂ (100 ml) and the mixture is cooled to 0°C. SOCl₂ (3.0 ml; 41.3 mmol) was added slowly over 15 min and the temperature was allowed to rise slowly to room temperature and stirred over night. The mixture was poured into stirring water (200 ml) and added CH₂Cl₂ (100 ml). The mixture was stirred for 30 min. The organic phase was washed with 5% NaH₂CO₃ (2×75 ml) and brine (2×75 ml) respectively, dried with sodium

sulfate and concentrated under reduced pressure. Recrystallized from toluene/ petroleum ether. Yield 6.75 g (97.8%).

(S)-(+)-2,2-dimethyl-1,3-dioxalane-4-methanol

5 Pulverized KOH (25 g) and 1-(Chloromethyl)-pyrene (6.0 g; 23.9 mmol) was added to a solution of (S)-(+)-2,2-dimethyl-1,3-dioxalane-4-methanol (2.6 g; 19.7 mmol) in dry toluene (250 ml). The mixture was refluxed under Dean-Stark conditions in 16h, then cooled to room temperature and added water (150 ml). The organic phase was washed with water (3×100 ml), dried with a combination of magnesium sulfate and sodium sulfate and
10 concentrated under reduced pressure to a thick oil. Silica gel chromatography (CH_2Cl_2) afforded the pure compound in 6.1 g (90%).

(R)-3-(1-Pyrenemethoxy)-propane-1,2-diol

15 (S)-(+)-2,2-dimethyl-1,3-dioxalane-4-methanol (6.1 g; 17.6 mmol) was added to a mixture of acetic acid and water (100 ml; 4:1) and is stirred at room temperature for 19h. Concentrated under reduced pressure. Giving an oil in quantitatively yield.

(S)-1-(4,4'-dimethoxytriphenylmethyloxy)-3-pyrenemethyloxy-2-propanol

20 (R)-3-(1-Pyrenemethoxy)-propane-1,2-diol (760 mg; 2.48 mmol) was dissolved in dry pyridine (20 ml) and added dimethoxytrityl chloride (920 mg; 2.72 mmol). The reaction mixture was stirred in 24h and concentrated under reduced pressure. Purified by silica gel chromatography (ethyl acetate/ cyclohexane/ triethylamine 49:49:2) to give a white foam. Yield 1.20 g (79.5%).

25 *Phosphoramidite of (S)-1-(4,4'-dimethoxytriphenylmethyloxy)-3-pyrenemethyloxy-2-propanol*

(S)-1-(4,4'-Dimethoxytriphenylmethyloxy)-3-pyrenemethyloxy-2-propanol (458 mg; 753 μmol), 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphan (453 mg; 429 μl ; 1.51 mmol) and diisopropylammonium tetrazolide (193 mg; 1.13 mmol) was mixed in dry CH_2Cl_2 (7 ml) and stirred under nitrogen atmosphere for 6 days. Purified by silica gel chromatography (ethyl

acetate/ cyclohexane/ triethylamine 49:49:2) and dried under reduced pressure. Yield 438 mg (72%) as a white foam.

Example 2

5 **Alternative synthesis procedure for 3-(1-Pyrenylmethoxy)-propane-1,2-diol**

1-Pyrenylmethanol (232 mg; 1.0 mmol) was dissolved in hot toluene (2 mL over Na). CsF (7 mg; 0,046 mmol) is added and stirred for approx. 1h at room temperature when 3-chloro-1,2-propandiol (170 mg; 1.53 mmol) is added. The mixture is stirred at 80°C for 2h, cooled off to room temperature and the precipitated product is separated from the mixture by
10 filtration. Washed with cold toluene (2 x 1 mL). Yield 220 mg (72%).

Synthesis is shown in scheme 1 of Figure 8.

Example 3

15 **Synthesis of the 2-O phosphoramidite of 1-O-4,4'-dimethoxytrityl-4-O-(9-antracenylmethyl)-1,2,4-butanetriol**

Schematic view of the synthesis of the 2-O- phosphoramidite of 1-O-4,4'-dimethoxytrityl-4-O-(9-antracenylmethyl)-1,2,4-butanetriol is shown in scheme 2 of Figure 9.

9-anthracenemethylchloride (II)

20 9-anthracenemethanol (0.81 g; 3.89 mmol; I) was dissolved in dry pyridine (467 µl; 5.83 mmol) and dry CH₂Cl₂. Under stirring and at 0°C SOCl₂ (423 µl; 5.83 mmol) was added dropwise, and the mixture was stirred for 24h during which the temperature is allowed to rise to room temperature within 2h. The reaction was poured onto stirring H₂O (60 ml) to which
25 was added additional CH₂Cl₂ (40 ml). The organic phase was washed with a 5% NaHCO₃ (100 ml) solution, brine (100 ml) and water (100 ml) respectively. Dried over Na₂SO₄ and concentrated *in vacuo*. Yield 665 mg (75%).

1,2-D- -isopropylidene-4-(9-anthracenylmethyl)-1,2,4-butanetriol (III)

9-anthracenemethylchlorid (628 mg; 277 mmol) was dissolved in dry toluene (25 ml over Na) and 2-[(S)-2',2'-dimethyl-1',3'-dioxolan-4'-yl]-ethanol (506 mg; 3.5 mmol) and 3 small spoons of KOH was added. The mixture was connected to a Dean-Stark apparatus and stirred under reflux conditions over night. The reaction mixture was slowly cooled to room temperature and washed with H₂O (4× 25 ml). Dried over Na₂SO₄ and concentrated *in vacuo*.

4-O-(9-anthracenylmethyl)-1,2,4-butanetriol (IV)

To the dried compound was added 80% AcOH (50 ml) and the reaction mixture was stirred in 24h at room temperature. The mixture was concentrated *in vacuo* over night and purified by short, fast silica gel chromatography (impurities were first eluted with CH₂Cl₂, and product was then eluted with 5% MeOH in CH₂Cl₂). Yield 56.3% over 2 steps.

1-O-(4,4'-dimethoxytrityl)-4-O-(9-anthracenylmethyl)-1,2,4-butanetriol (V)

The diol (425 mg; 1.40 mmol) and DMT-Cl was mixed in dry pyridine (5 ml) and stirred at room temperature for 36h. The reaction mixture was concentrated *in vacuo* and purified by silica gel chromatography (EtOAc: C₆H₁₂:N(Et)₃ 63:35:2). Co-evaporated with ether (5 ml over Na) after which a yellowish foam was isolated. Yield 630 mg (74%).

Phosphoramidite of 1-O-(4,4'-dimethoxytrityl)-4-O-(9-anthracenylmethyl)-1,2,4-butanetriol (VI)

The DMT protected anthracene compound was dissolved in dry CH₂Cl₂ (7 ml) and diisopropylammonium tetrazolide (252 mg; 1.5 mmol) and 2-Cyanoethyl N,N,N',N'-tetraisopropyl Phosphane was added. The reaction mixture was stirred for 20h at room temperature. Concentrated *in vacuo* and purified by silica gel chromatography (EtOAc: C₆H₁₂:N(Et)₃ 24:74:2). Co-evaporated with ether (5 ml over Na) to give a yellowish foam (67%).

Example 4

Synthesis of the phosphoramidite of (S)-1-(4,4'-dimethoxytriphenylmethoxy)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-2-butanol (V)

Synthesis of the phosphoramidite of (S)-1-(4,4'-dimethoxytriphenylmethoxy)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-2-butanol (V) is shown in Figure 10.

7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one (I)

7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one was prepared according to literature procedures (Schmidt, U. & Kubitzek, H., Chem. Ber., 93, 1559 (1960); Hassan, K. M. et al. Phosphorous, Sulfur, Silicon Relat. Elem., 47, 181 (1990); Gewald, K. & Jänsch, H. J., Prakt. Chemie 313-320 (1976).

3-N-((S)-2'',2''-dimethyl-1'',3''-dioxalane-4''-ethanyl)-7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one (II)

7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one (1.16 g; 5.0 mmol) was suspended in anhydrous DMF (20 ml) and NaH (0.2 g; 5.0 mmol, 60% dispersion in mineral oil) was added. The mixture was stirred for 2h until all H₂ evolving ceased. Then (S)-2,2-dimethyl-1,3-dioxalane-4-ethanoyl-O-para-toluenesulfonate (0.78 g; 5.1 mmol) was added in one portion and the mixture was stirred for 24h at 80°C. The mixture was evaporated to dryness *in vacuo*, co evaporated with dry toluene (3 × 10 ml) *in vacuo* and the residue was purified silica gel chromatography (5% EtOAc in CHCl₃) to get a colorless product. Yield 0.81 g; 45%.

(S)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-butan-1,2-diol (III)

3-N-((S)-2'',2''-dimethyl-1'',3''-dioxalane-4''-ethanyl)-7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one (0.75 g; 2.1 mmol) was stirred at room temperature in 80% AcOH (20 mL) for 24h. The product was obtained by concentration *in vacuo* and co-evaporation with EtOH. Purified by silica gel chromatography (5% MeOH in CHCl₃) to get the colorless product. Yield 0.5 g (75%).

(S)-1-(4,4'-dimethoxytriphenylmethyloxy)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-2-butanol (IV)

(S)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-butan-1,2-diol (0.6 g; 1.9 mmol) was dissolved in dry pyridine (5 ml) and DMT-Cl (0.71 g; 2.1 mmol) was added. Stirred at room temperature over night. Concentrated *in vacuo* and co evaporated using dry toluene (3 × 10 ml). The residue was purified by silica gel chromatography (EtOAc: $C_6H_{12}:N(Et)_3$ 49:49:2) to yield a white foam. Yield 0.77 g (65%)

Phosphoramidite of (S)-1-(4,4'-dimethoxytriphenylmethyloxy)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-2-butanol (V)

(S)-1-(4,4'-dimethoxytriphenylmethyloxy)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-2-butanol (310 mg; 0.5 mmol) was dissolved under nitrogen in anhydrous dichloromethane (10 ml). Diisopropylammoniumtetrazolide (0.11 g; 0.67 mmol) was added followed by dropwise addition of 2-Cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite (0.3 g; 1.0 mmol) the reaction was stirred over night under nitrogen atmosphere, concentrated *in vacuo* and purified by silica gel chromatography (EtOAc: $C_6H_{12}:N(Et)_3$ 49:45:12) to give a white foam. Yield 345 mg (84%).

Methylation

The amount or degree of methylation of genomic DNA has implications in many conditions such as aging, stem cell differentiation, genetic abnormalities, cancer and other disease states. A number of important implications of methylation states were set out below.

The fusion of Embryonic Stem Cells with adult thymocytes to examine the reprogramming that occurs at the level of DNA methylation after the fusion has been made. The inactive somatic X becomes activated as visualized by whole chromosome examination (Tada *et al.*, 2001; Current Biology, 11, 1553-1558).

Examination of methylation patterns in specific DNA regions in the clinicopathological features of sporadic colorectal cancers; as an inexpensive and accurate way of identifying such tumors (Ward *et al.*, 2001; Gut, 48,821-829), and the methylation patterns in stem cells

in human colon crypts (Ro et al., 2001, Proc Natl Acad Sci, USA, 98, 10519-10521; Yatabe et al., 2001, Proc. Natl. Acad Sci USA, on line edition).

Methylation patterns in prostate cancer, and in cell lines treated with 5-azacytidine in order to reactivate specific genes (Chetcuti et al., 2001, Cancer Research, 61, 6331-6334):

5 Methylation patterns in the various Estrogen receptors in uterine endometrial cancers where gene inactivation via methylation occurs in many cancers but is not at a high frequency in normal individuals (Sasaki et al., 2001, Cancer Research, 61, 3262-3266).

Methylation patterns in bladder cancer (Markl et al., 2001, Cancer Research, 61, 5875-5884).

10 Methylation patterns in breast cancer (Nielsen et al., 2001, Cancer Letters, 163, 59-69).

Methylation patterns in specific promoters involved in lung and breast cancers (Burbee et al., 2001, J Natl Cancer Institute, 93, 691-699).

15 Methylation patterns in free DNA in the plasma of patients with esophageal adenocarcinomas (Kawakami et al., 2000, J Natl Cancer Institute, 92, 1805-1811).

Methylation of the CDH1 promoter in hereditary diffuse gastric cancer (Grady et al., 2000, Nature Genetics, 26, 16-17).

20 Genomic imprinting, in which, for example, a paternal allele of a gene is active, and the maternal allele is inactive, or vice versa. This inactivation is accomplished via methylation changes in the genes involved, or in sequences nearby to them. In essence, DNA regions become methylated in the germ line of one sex, but not in that of another (Mann, 2001, Stem Cells, 19, 287-294).

25 Genome-wide methylation patterns in studies of cloning of various species (sheep, cattle, goats, pigs and mice), via nuclear transfer or *in vitro* fertilization. Thus the methylation patterns of donor nuclei that were inserted into oocytes vary greatly, and this is thought to be the reason why there is such a high failure rate in current cloning experiments. These differentiated nuclei probably require more reprogramming than less differentiated ones such as in Embryonic Stem Cells (Kang et al, 2001; Nature Genetics, 28, 173-177; Humphreys et al., 2001, Science, 293, 95-97).

30 Excessive hyper-methylation patterns in 24 cancer cell lines versus normal tissues (Smiraglia et al., 2001, Human Molecular Genetics, 10, 1413-1419).

Insertion of methylated DNA into a non methylated mini gene construct to examine the effects on gene expression and imprinting (Holmgren et al., 2001, *Current Biology*, 11, 1128-1130).

5 Methylation patterns in mature B cell lymphomas, where specific genes were inactivated by methylation (Malone et al., 2001, *Proc Natl Acad Sci USA* 98, 10404-10409).

Methylation patterns of particular genes in acute myeloid leukemia (Melki et al., 1999, *Leukemia*, 13, 877-883).

10 Analysis of the *Mecp2* gene in knockout mice: This protein is involved in binding to methylated sites in DNA and is thought to be involved in Rett syndrome, which is an inherited neurological disorder (Guy et al., *Nature Genetics*, 27, 322-326).

Methylation patterns of 5 specific genes during the normal aging process, and in ulcerative colitis (Issa et al., 2001, *Cancer Research*, 61, 3573-3577).

15 Loss of methylation in the processes of apoptosis, which impinge upon signal transduction pathways, cell cycle control, movement of mobile elements within the genome (Jackson-Grusby et al., 2001, *Nature Genetics*, 27, 31-39).

Comparison of the methylation patterns of promoter and gene regions in different species, such as human and mouse, to determine the evolutionary conservation or lack thereof of CpG islands involved in gene regulation (Cuadrado et al., 2001, *EMBO Reports*, 21, 586-592).

20 DNA methylation patterns in testicular sperm at different developmental stages (Manning et al., 2001, *Urol Int*, 67, 151-155).

Immuno histochemical staining using a monoclonal antibody to analyze DNA methylation patterns (Piyathilake et al., 2000, *Biotechnic and Histochem*, 75, 251-258).

25 Differences between the methylation patterns of genes and pseudogenes (Grunau et al., 2000, *Human Mol Genet*, 9, 2651-2663).

5-methylcytosine content of model invertebrates such as *Drosophila melanogaster* (Gowher et al., 2000, *EMBO J*, 19, 6918-6923).

Large scale mapping of human promoters using the methylation patterns of CpG islands (Ioshikhes et al, 2000, *Nature Genetics*, 26, 61-63).

Induced changes in the processes of chromatin remodelling, DNA methylation and gene expression during mammalian development due to changes in the expression of the ATRX gene which give rise to mental retardation, facial dysmorphism, urogenital abnormalities and alpha thalassemia (Gibbons et al., 2000, *Nature Genetics*, 24, 368-371).

- 5 Boundaries between methylated and unmethylated domains in the promoter region of the GSTP1 gene involved in prostate cancer (Millar et al., 2000, *J Biological Chemistry*, 275, 24893-24899; Millar et al., 1999, *Oncogene*, 18, 1313-1324).

Methylation changes during the normal processes of aging (Toyota et al., 1999, *Seminars in Cancer Biology*, 9, 349-357).

- 10 Methylation changes in aging and in atherosclerosis in the cardiovascular system, (Post et al., 1999, *Cardiovascular Research*, 43, 985-991) and during normal aging and cancers in colorectal mucosa (Ahuja et al., 1998, *Cancer Research*, 58, 5489-5494).

Methylation patterns in germ cells and sertoli cells in testis (Coffigny et al., 1999, *Cytogenet Cell Genets*, 87, 175-181).

- 15 DNA methylation changes during the development of model vertebrates such as the zebrafish (Macleod et al., 1999, *Nature Genetics*, 23, 139-140).

Methylation patterns in the promoter regions of the human histo-blood ABO genes (Kominato et al., 1999, *J Biol Chem*, 274, 37240-37250).

- 20 Methylation patterns during mammalian preimplantation development using monoclonal antibodies (Rougier et al., 1999, *Genes and Development*, 12, 2108-2113).

Methylation patterns induced by various cancer chemotherapeutic drugs (Nyce, 1997, *Mutation Research*, 386, 153-161; Nyce 1989, *Cancer Research*, 49, 5829-5836) and the changes in DNA methylation in phenobarbital-induced and spontaneous liver tumors (Ray et al., 1994, *Molecular Carcinogenesis* 9, 155-166).

- 25 Analysis of 5-methylcytosine residues in DNA by the bisulfite sequencing method (Grigg, 1996, *DNA Sequence*, 6, 189-198).

Isolation of CpG islands using a methylated DNA binding column (Cross et al., 1994, *Nature Genetics*, 6, 236-244).

Is KSHV lytic growth induced by a methylation-sensitive switch? (Laman and Boshoff, Trends Microbiol 2001 Oct; 9(10):464-6). Both latent and lytic growth of Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8) contribute to its pathogenesis.

As can be seen from the large number of examples of different methylation states and implications provided above, it will be appreciated that the present invention offers a powerful tool for the study of methylation and thus is useful for many aspects of disease and health.

Table 1 shows some examples of solid supports useful for attaching capture ligands of the present invention. Table 2 shows possible choices of detector systems for use in the present invention.

Table 1 Solid supports for attachment of capture ligands

label	fluoro bead	column	magnetic bead	latex bead	p/styrene bead	membrane	glass
INA	+	+	+	+	+	+	+
Oligo	+	+	+	+	+	+	+
RNA	+	+	+	+	+	+	+
Chimer a	+	+	+	+	+	+	+

Table 2 Detection systems for detection ligands

Label	Fluoro Bead	Magnetic bead	latex bead	p/styrene bead	Glass	Aptamer
pre-label	+					
fluoresence	+	+	+	+	+	+
radiolabel	+	+	+	+	+	+
Dendrimer	+	+	+	+	+	+

MATERIALS AND METHODS

Intercalating Nucleic Acids (INAs)

Intercalating nucleic acids (INAs) are non-naturally occurring polynucleotides which can hybridize to nucleic acids (DNA and RNA) with sequence specificity. INAs are candidates as alternatives/substitutes to nucleic acid probes in probe-based hybridization assays because they exhibit several desirable properties. INAs are polymers which hybridize to nucleic acids to form hybrids which are more thermodynamically stable than a corresponding nucleic acid/nucleic acid complex. They are not substrates for the enzymes which are known to degrade peptides or nucleic acids. Therefore, INAs should be more stable in biological samples, as well as, have a longer shelf-life than naturally occurring nucleic acid fragments. Unlike nucleic acid hybridization which is very dependent on ionic strength, the hybridization of an INA with a nucleic acid is fairly independent of ionic strength and is favoured at low ionic strength under conditions which strongly disfavour the hybridization of nucleic acid to nucleic acid. The binding strength of INAs is dependent on the number of intercalating groups engineered into the molecule as well as the usual interactions from hydrogen bonding between bases stacked in a specific fashion in a double stranded structure. Sequence discrimination is more efficient for INA recognizing DNA than for DNA recognizing DNA.

INAs are synthesized by adaptation of standard oligonucleotide synthesis procedures in a format which is commercially available.

There are indeed many differences between INA probes and standard nucleic acid probes. These differences can be conveniently broken down into biological, structural, and physico-chemical differences. As discussed above and below, these biological, structural, and physico-chemical differences may lead to unpredictable results when attempting to use INA probes in applications where nucleic acids have typically been employed. This non-equivalency of differing compositions is often observed in the chemical arts.

With regard to biological differences, nucleic acids are biological materials that play a central role in the life of living species as agents of genetic transmission and expression. Their *in vivo* properties are fairly well understood. INA, however, is a recently developed totally artificial molecule, conceived in the minds of chemists and made using synthetic organic chemistry. It has no known biological function.

Structurally, INA also differs dramatically from nucleic acids. Although both can employ common nucleobases (A, C, G, T, and U), the composition of these molecules is structurally diverse. The backbones of RNA, DNA and INA are composed of repeating phosphodiester ribose and 2-deoxyribose units. INAs differ from DNA or RNA in having one or more large flat molecules attached via a linker molecule(s) to the polymer. The flat molecules intercalate between bases in the complementary DNA stand opposite the INA in a double stranded structure.

The physico/chemical differences between INA and DNA or RNA are also substantial. INA binds to complementary DNA more rapidly than nucleic acid probes bind to the same target sequence. Unlike DNA or RNA fragments, INAs bind poorly to RNA unless the intercalating groups are located in terminal positions. Because of the strong interactions between the intercalating groups and bases on the complementary DNA strand, the stability of the INA/DNA complex is higher than that of an analogous DNA/DNA or RNA/DNA complex.

Unlike other DNA such as DNA or RNA fragments or PNAs, INAs do not exhibit self aggregation or binding properties.

In summary, because INAs hybridize to nucleic acids with sequence specificity, INAs are useful candidates for developing probe-based assays. However, INA probes are not the equivalent of nucleic acid probes. Consequently, any method, kits or compositions which could improve the specificity, sensitivity and reliability of probe-based assays would be useful in the detection, analysis and quantitation of DNA containing samples. INAs have the necessary properties for this purpose.

Sodium bisulfite – a specific deamination method

Standard methods for treating nucleic acid with sodium bisulfite can be found in a number of references including Frommer et al 1992, Proc Natl Acad Sci 89:1827-1831; Grigg and Clark 1994 BioAssays 16:431-436; Shapiro et al 1970, J Amer Chem Soc 92:422 to 423; Wataya and Hayatsu 1972, Biochemistry 11:3583 - 3588. Some improvements to these protocols have also been developed by the present inventors.

Detection systems

Coating Magnetic beads

The INA, DNA, LNA, HNA, ANA, MNA used for attachment to the magnetic beads can be modified in a number of ways. In this example, the INA contained either a 5' or 3' amino group for the covalent attachment of the INA to the beads using a hetero-bifunctional linker such as is used EDC. However, the INA can also be modified with 5' groups such as biotin which can then be passively attached to magnetic beads modified with avidin or streptavidin groups.

Ten μ l of carboxylate modified Magnabind™ beads (Pierce) or 100 μ l of Dynabeads™ Streptavidin (Dyna) were transferred to a clean 1.5 ml tube and 90 μ l of PBS solution added to the magnetic beads.

The beads were mixed then magnetised and the supernatant discarded. The beads were washed x2 in 100 μ l of PBS per wash and finally resuspended in 90 μ l of 50 mM MES buffer pH 4.5 or another buffer as determined by the manufactures' specifications.

One μ l of 250 μ M INA, DNA, LNA, HNA, ANA, MNA (concentration dependant on the specific activity of the selected INA as determined by oligonucleotide hybridisation experiments) is added to the sample and the tube vortexed and left at room temperature for 10-20 minutes.

Ten μ l of a freshly prepared 25 mg/ml EDC solution (Pierce/Sigma) is then added, the sample vortexed and incubated at either room temperature or 4°C for up to 60 minutes.

The samples were then magnetised, the supernatant discarded and the beads, if necessary, be blocked by the addition of 100 μ l either 0.25 M NaOH or 0.5 M Tris pH 8.0 for 10 minutes.

The beads were then washed x2 with PBS solution and finally resuspended in 100 μ l PBS solution.

Hybridisation using the magnetic beads

Ten μ l of INA coated Magnabind™ beads were transferred to a clean tube and 40 μ l of either ExpressHyb™ buffer (Clontech) either neat or diluted 1:1 in distilled water or any other commercial or in-house hybridization buffer. The buffers may also contain either

cationic/anionic or zwittergents at known concentration or other additives such as Heparin and poly amino acids.

Heat denatured sample of DNA 1-5 μ l was then added to the above solution and the tubes vortexed and then incubated at 55°C or another temperature depending on the melting temperature of the chosen INA for 20-60 minutes.

The samples were magnetised and the supernatant discarded and the beads washed x2 with 0.1XSSC/0.1%SDS at the hybridisation temperature from earlier step for 5 minutes per wash, magnetising the samples between washes.

10 *Dual INA capture*

INA#1 was coupled to a carboxylate modified magnetic bead via a N- or C-terminal amine of the INA and washed to remove unbound INA.

The INA/bead complex is then hybridised to the target DNA in solution using appropriate hybridisation and washing conditions.

15 The target DNA was then released from the magnetic bead using appropriate methods and transferred to a tube containing a second INA/magnetic beads complex targeted to the opposite end of the DNA molecule.

The second INA/bead complex or oligo/bead complex was then hybridised to the target DNA in solution using appropriate hybridisation and washing conditions.

20 A third INA or oligonucleotide complementary to the central region of the target DNA could be used as a detector molecule. This detector molecule can be labelled in a number of ways.

25 (i) The INA, DNA, LNA, HNA, ANA, MNA can be directly labelled with a radioactive isotope such as P^{32} or I^{125} and then hybridised with the target DNA.

(ii) The INA, DNA, LNA, HNA, ANA, MNA can be labelled with a fluorescent molecule such as Cy-3 or Cy-5 and then hybridised with the target DNA.

30 (iii) An amine modified INA, DNA, LNA, HNA, ANA, MNA can be labelled in either of the above ways then coupled to a carboxylate modified microsphere of known size then the sphere washed to remove unbound labelled INA, PNA or

oligo. This bead complex can then be used to produce a signal amplification system for the detection of the specific DNA molecule.

- (iv) The INA, DNA, LNA, HNA, ANA, MNA can be attached to a dendrimer molecule either labelled with fluorescent or radioactive groups and this complex used to produce a signal amplification.
- (v) The INA, DNA, LNA, HNA, ANA, MNA labelled in any of the above ways and hybridised to the target DNA on a solid support can be released into solution using a single stranded specific nuclease such a mung bean nuclease or S1 nuclease. The released detector molecule can be read in a suitable device.

Preparation of radio-labelled detector spheres

An INA, DNA, LNA, HNA, ANA, MNA can be either 3' or 5' labelled with a molecule such as an amine group, thiol group or biotin.

The labelled molecule can also have a second label such as P^{32} or I^{125} incorporated at the opposite end of the molecule to the first label.

This dual labelled detector molecule can be covalently coupled to a carboxylate or modified latex bead for example of known size using a hetero-bifunctional linker such as EDC. Other suitable substrates can also be used depending on the assay.

The unbound molecules can then be removed by washing leaving a bead coated with large numbers of specific detector/signal amplification molecules.

These beads can then be hybridised with the DNA sample of interest to produce signal amplification.

Preparation of fluorescent labelled detector spheres

An INA, DNA, LNA, HNA, ANA, MNA can be either 3' or 5' labelled with a molecule such as an amine group, thiol group or biotin.

The labelled molecule can also have a second label such as Cy-3 or Cy-5 incorporated at the opposite end of the molecule to the first label.

This dual labelled detector molecule can now be covalently coupled to a carboxylate or modified latex bead of known size using a hetero-bifunctional linker such as EDC.

The unbound molecules can then be removed by washing leaving a bead coated with large numbers of specific detector/signal amplification molecules.

These beads can then be hybridised with the DNA sample of interest to produce signal amplification.

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Preparation of enzyme labelled detector spheres

An INA, DNA, LNA, HNA, ANA, MNA can be either 3' or 5' labelled with a molecule such as an amine group or a thiol group.

10 The labelled molecule can also have a second label such as biotin or other molecules such as horse-radish peroxidase or alkaline phosphatase conjugated on via a hetero-bifunctional linker at the opposite end of the molecule to the first label.

This dual labelled detector molecule can now be covalently coupled to a carboxylate or modified latex bead of known size using a hetero-bifunctional linker such as EDC.

15 The unbound molecules can then be removed by washing leaving a bead coated with large numbers of specific detector/signal amplification molecules.

These beads can then be hybridised with the DNA sample of interest to produce signal amplification.

Signal amplification can then be achieved by binding of a molecule such as streptavidin or an enzymatic reaction involving a colorimetric substrate.

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INA oligomer combinations

In all of the above cases the initial hybridization event involved the use of magnetic beads coated with an INA complementary to the DNA of interest.

The second hybridisation event can involve any of the methods mentioned above.

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This hybridisation reaction can be done with either a second INA complementary to the DNA of interest, a PNA or an oligonucleotide or modified oligonucleotide complementary to the DNA of interest. As fluorescent beads of convenient size in these assays, carry $>10^6$ fluorochrome molecules and a single fluorescent bead can be detected readily, the method has the potential sensitivity to assay one or a few DNA molecules from one or a few cells.

Dendrimers and aptamers

Dendrimers are branched tree-like molecules that can be chemically synthesised in a controlled manner so that multiple layers can be generated that were labelled with specific molecules. They were synthesised stepwise from the centre to the periphery or visa-versa.

One of the most important parameters governing dendrimer structure and its generation is the number of branches generated at each step; this determines the number of repetitive steps required to build the desired molecule.

Dendrimers can be synthesised that contain radioactive labels such as I^{125} or P^{32} or fluorescent labels such as Cy-3 or Cy-5 to enhance signal amplification.

Alternatively dendrimers can be synthesised to contain carboxylate groups or any other reactive group that could be used to attach a modified INA, PNA or DNA molecule.

METHODS

Figure 11 and Figure 12 show examples of the method of the invention using sandwich INA signal amplification using solid supports and magnetic beads, respectively. Although INA is exemplified as the ligand in Figure 11 and Figure 12, it will be appreciated that other capture or detector ligands such as oligonucleotides can be used in these methods.

A solid support in the form of a microfilter well was provided and coated with N-oxysuccinimide to assist in the adhesion of INA or other ligand to the well.

A first INA which was complementary to a first part of the target nucleotide sequence is added to the well and attached to this solid support.

Bisulfite treated DNA was then added to the well and allowed to hybridise with the INA to capture the target DNA which had hybridised to the INA and subsequently bound to the well.

The well was then washed to remove the hybridisation solution and any non-hybridised DNA leaving only the hybridised DNA captured on the well.

Next a second INA, which was complementary to a second part of the target nucleotide sequence was linked to microsphere beads having fluorescent labelling. The

second linked INA was then hybridised with the target DNA already bound to the well. The well was then washed to remove the unhybridized second INA/microsphere complex leaving only the INA/microsphere complex and fluorescent label associated with the target DNA sequence.

5 The fluorescence was then measured to determine the level of target DNA.

Detection of methylated DNA using microspheres

Methodology

10 Referring to Figure 11 and Figure 12, the detection of methylated DNA using microspheres is shown.

Coating Microtitre wells with capture INA

- 15 (i) The capture INA (0.0-100 pM per well) in 50 mM Phosphate buffer, 1 mM EDTA pH 8.5 (100 µl) was used to coat N-oxy succinimide-coated microtitre wells (Costar Cat#2498) for 16-24 hours @ 4°C.
- (ii) Plates were washed with 100 µl of 50 mM Phosphate buffer, 1 mM EDTA pH 8.5.
- (iii) 150 µl of 3% BSA, 50 mM Phosphate buffer, 1 mM EDTA pH 8.5 was added to each well and the plates left @ 4°C until required.

Coating the Fluorospheres with detection INA

- 20 (i) Fluorospheres (Molecular Probes) were sonicated five times for 5 seconds to break up any aggregated material.
- 25 (ii) The detection probe INA was diluted in a range from 300 pM to 0.3 pM in 250 µl of sonicated 50 mM 2[N-morpholino] ethanesulphonic acid (MES) pH 6.0 and 250 µl of sonicated fluorospheres added and the solution left at room temperature for 30 minutes.

- (iii) 0.5 mg of 1-ethyl-3[3 dimethylamine propyl] carbodiimide [EDAC], Sigma Cat #E1769, was added to the sample and the sample left 4-6 hours at room temperature in the dark then incubated 16 hours at 4°C.
- (iv) 55 µl of 1M glycine was added to the beads and the beads left at room temperature for 2 hours.
- (v) The beads were centrifuged for 5-20 minutes (dependant on size of beads, generally 0.5 µM beads required 5 mins while 0.1 µM beads required 20 minutes) at 14,000 rpm in a bench top centrifuge and the supernatant discarded.
- (vi) Beads were washed twice with 500 µl of PBS/1% BSA with centrifugation as before between wash steps.
- (vii) The beads were then resuspended in 200 µl of PBS/1% BSA and stored at 4°C in the dark until required.
- (viii) Variation of the number of INAs bound to the beads can be used to optimise sensitivity and minimise background levels.

Hybridisation of DNA

- (i) Either control salmon sperm DNA or DNA that was bisulfite treated as in Clark *et al* (Clark SJ, Harrison J, Paul CL and Frommer M. High sensitivity mapping of methylated cytosines. Nucleic Acids Res. 22: 2990-2997 (1994)) was hybridised with INAs coupled to microtitre wells then added to per well.
- (ii) DNA samples were mixed with 100 µl of ExpressHyb™ buffer (Clontech), added to the wells and the plate covered with cling film or the wells overlaid with mineral oil (Sigma) for longer incubations and the samples incubated at between 45-60°C for between 1-16 hours.
- (iii) Wells were then washed twice with 150 µl of 2X SSC/0.1%SDS @ 45-60°C for 5-10 minutes per wash.
- (iv) The wells were further washed with 150 µl of 0.1X SSC/0.1%SDS @ 45-60°C for 5-10 minutes and the wash solution discarded.

- (v) The INA/fluorospheres were diluted 1/100 in ExpressHyb™ buffer (Clontech) and 100 µl of samples added to the wells. The plates were covered with cling film or the wells overlayed with mineral oil (Sigma) for longer incubations and the samples incubated @ between 45-60°C for between 1-16 hours.
- (vi) Wells were then washed twice with 150 µl of 2X SSC/0.1%SDS at 45-60°C for 5-10 minutes per wash.
- (vii) The wells were further washed with 150 µl of 0.1X SSC/0.1%SDS at 45-60°C for 5-10 minutes and the wash solution discarded.
- (viii) Finally the fluorescent intensity of each well was measured at the appropriate excitation/emission wave-length for the particular bead (500/520 for yellow beads) in a Victor II fluorescent plate reader.
- (ix) Background values measured in wells to which no INA had been attached were subtracted from all readings.

15 **Method for the production of in-house coated radiolabelled beads**

- (i) A specific oligonucleotide (or INA) is synthesised against the target DNA region of interest. This oligonucleotide contains a 3' amine group synthesised using standard chemistry (Sigma Genosys).
- (ii) The oligonucleotide (or INA) was then 5' kinased using gamma P³²dATP as follows:
- Oligonucleotide (20 ng/µl) 1 µl
 - X10 PNK buffer 1 µl
 - T4 PNK 1 µl
 - Gamma P³²dATP 2 µl
 - Sterile water 5 µl
- (iii) The sample was then incubated at 37°C for 1 hour then heated to 95°C for 5 minutes to inactivate the enzyme.

- (iv) 0.1 μ M carboxylate modified fluorescent beads (Molecular Probes Cat# F-8803) are diluted 1/10,000, 1/100,000 and 1/1,000,000 in sterile water then the kinased oligonucleotide coupled to the beads as follows:

- Beads 1 μ l
- Labelled oligo 3 μ l
- 50 mM MES pH 8.0 5 μ l
- 10 mg/ml EDC (Pierce) 2 μ l

- (v) The beads were then incubated @ room temperature for 1 hour to allow the kinased oligonucleotide to attach to the beads via the 3' amine.

- (vi) The beads were then spun in a microfuge at full speed for 15 minutes to sediment the coated beads.

- (vii) The supernatant was removed and the beads washed with 100 μ l of PBS solution and spun as above.

- (viii) The supernatant was removed and the beads resuspended in 50 μ l of PBS.

- (ix) The CPM of the coated beads was then measured in a standard scintillation counter using the Cerenkov counting protocol. The beads with the highest activity were then used as a detector system in the assay

The idea behind this protocol was to produce the smallest number of beads with the highest specific activity, so that only a few beads are needed to bind to the target sequence in order to generate a detectable signal.

Urea and methoxyamine conditions of use

- (i) Typically 2 μ g of genomic DNA is restriction digested with an appropriate enzyme (as determined by the target DNA sequence) under the manufacturers conditions for at least 2 hours in a final volume of 20 μ l.
- (ii) 2.2 μ l of freshly prepared 3 M NaOH (6g in 50 ml H₂O) is added to the DNA and the sample incubated @ 37°C for 15 minutes.
- (iii) 6.24 g of urea is added to 10 ml of sterile distilled water and the solution mixed until gently until the urea has dissolved.

- (iv) 7.6 g of sodium metabisulphite (BDH Analar™) is then added and again the solution mixed gently until the bisulphite had dissolved.
- (v) The pH of the reagent is then adjusted to 5.0 with 10 M NaOH and the volume of the reagent made to 20 ml with sterile water.
- 5 (vi) 208 µl of the reagent is then added to the digested denatured genomic DNA sample.
- (vii) 12 µl of 10-100 mM quinol is added the solution mixed and overlayed with mineral oil and incubated for 16 hours at 55°C in the dark.
- 10 (viii) The mineral oil is then removed and the DNA purified using the Promega Wizard™ DNA purification system according to the manufacturers instructions.
- (ix) The DNA is eluted from the column with 50 µl of sterile water then 5.5 µl of 3 M NaOH added and the sample incubated at 37°C for 15 minutes.
- 15 (x) At this stage 1/10 volume of methoxyamine (Sigma) from 1-100 mM can be added and incubated with the NaOH as an agent to minimise the nicking of the bisulphite treated DNA.
- (xi) In addition tRNA can or glycogen may be added at this stage to help precipitate the DNA.
- 20 (xii) The DNA is then precipitated by the addition of 33.5 µl of 5 M NH₄OAcetate pH 7.0 and 330 µl of 100% ethanol.
- (xiii) The samples are incubated at -20°C for at least 1 hour then spun down in a microcentrifuge at full speed for 15 minutes
- 25 (xiv) The pellet is then air dried for 5-10 minutes and the DNA resuspended in 10 mM Tris/0.1 mM EDTA pH 8.0 in volumes ranging from 10-100 µl dependant on the downstream processing of the modified DNA.

Variations on the above protocol are set out below.

If very small quantities of DNA or micro-dissected cells are to be bisulfite treated this can be done in a number of ways.

- Restriction digestion can be omitted.

- Urea can be omitted
 - Glycogen or tRNA or a combination of both can be added at steps (iv), (viii) and (x).
 - The bisulfite reaction can be done by encapsulating the DNA to be modified in agarose bead, and the entire reaction carried out while the DNA is in the bead.
- 5 • The time of the reaction with the bisulphite can be reduced from 16 hours to as little as 1 hour but more usually 4 hours.

SUMMARY

10 The methods of the present invention can be applied for the detection of any DNA using one ligand (preferably an oligonucleotide or INA) bound to a solid support and one coupled to a microsphere. Natural oligonucleotides or INAs may be used, but INAs were preferred because of their specificity, stability and rate of hybridisation.

15 In one particular adaptation, the methods of the invention can be used to distinguish the presence of methylated cytosines in DNA that has been treated with sodium bisulfite. The specificity of hybridisation can be used to discriminate against molecules that have not reacted completely with bisulfite (one or more cytosines not converted to uracil) as well as distinguishing between methylated cytosines at CpG sites (which remain as cytosines) and unmethylated CpG sites where the cytosine is converted to uracil.

20 In another adaptation the methods of the invention can be used to discriminate against DNA whose cytosines have not reacted completely with bisulfite reagent to convert them to uracils because they happen to carry a methyl group in the 5' position.

25 As treatment with bisulfite changes the sequence of the DNA by converting all cytosines (but not 5-methyl cytosines) to uracils, specific INAs can be made which recognise a region having 5 methyl cytosines but which will not recognise the same sequence which happens to have no 5-methyl cytosines.

The methods of the invention can also be applied to the discrimination of different alleles of a gene where the sequence of one or both of the oligonucleotides or INAs will match perfectly with one allele but mismatch with the other.

The method of the invention has numerous applications as previously described including particular use in devising multiple array chips for rapid detection of the methylation status of bulk DNA samples.

5 It will be appreciated that the methods are applicable for many other states and conditions where different methylation states have been found to play a role in disease or altered state of cells. Examples of just some genes affected by CpG methylation are shown in Table 3. The present invention is clearly applicable for the detection or measurement of such methylation states and many others.

Table 3 Examples of genes affected by CpG or CpNpG methylation

Gene	Location	Cancer	Aging	Comments
APC	5q21	Colon, gastric, oesophageal	No	
BRaC-1	17q21	Breast, ovarian	No	
Calcitonin	11p15	Colon, lung, haematological	No	One of the first to be found methylated in cancer
E-cadherin	16q22.1	Breast, gastric, thyroid, SCC, leukemia, liver	No	
Estrogen Receptor	6q25.1	Colon, liver, heart, breast, lung	Yes	Good correlation between methylation and loss of expression
H19	11p15.5	Wilms tumour	No	Imprinted gene
HIC1	19p13.3	Prostate, breast, brain, lung	Yes	Candidate tumour suppressor
IGF2	11p15.5	Colon, AML	Yes	Has large CpG island
MDGI	1p33-35	Breast	No	
MGMT	10q26	Brain, colon, lung, breast	No	
MYOD1	11p15.4	Colon, breast, bladder, lung	Yes	

Table 3 cont'd Examples of genes affected by CpG or CpNpG methylation

Gene	Location	Cancer	Aging	Comments
N33	8p22	Colon, prostate, brain	Yes	Oligo-saccharyl-transferase
P15	9q21	Leukemia, lung, colon	No	
P16	9q21	Lung, colon, lymphoma, bladder, and more	No	Methylation occurs as frequent as deletions or other mutations
TIMP3	22q12.1	Brain, kidney	No	
WT1	11p13	Breast, colon, Wilms tumour	No	

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present
5 embodiments were, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this 24th day of January 2003

Human Genetic Signatures Pty Ltd

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PATENT & TRADE MARKS ATTORNEYS

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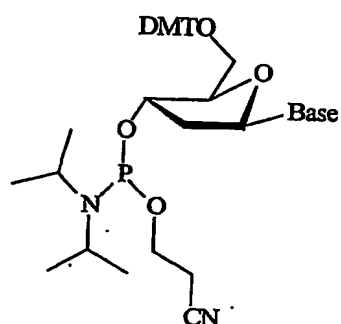
Yamana K, Takei M and Nakano H, 1997, Synthesis of oligonucleotide derivatives containing pyrene labelled glycerol linkers: enhanced fluorescence on binding to a complementary DNA sequence; Tetrahedron Letters, 38:6051-6054.

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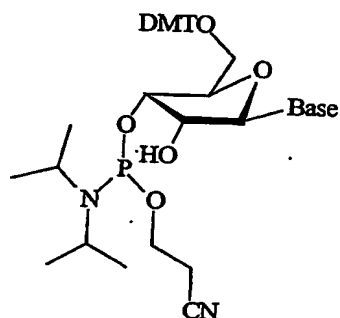
Figure 1. Range of different backbone monomer units of nucleotides and nucleotide analogues, and how they are connected to the nucleobases via linkers that are attached at one or two positions of the backbone monomer unit.

Examples of oligomers of DNA, RNA & PNA

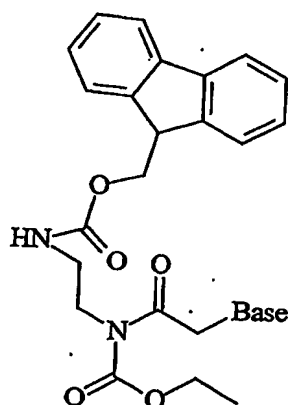
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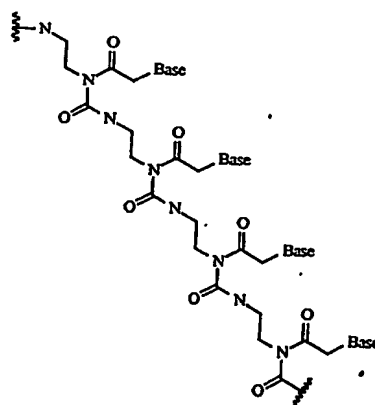
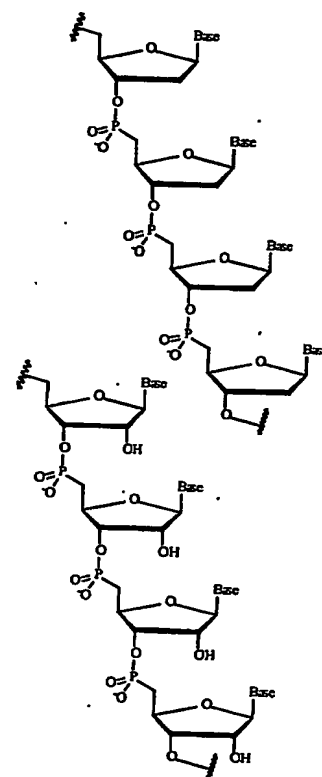
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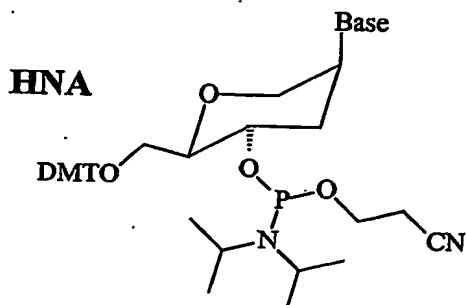
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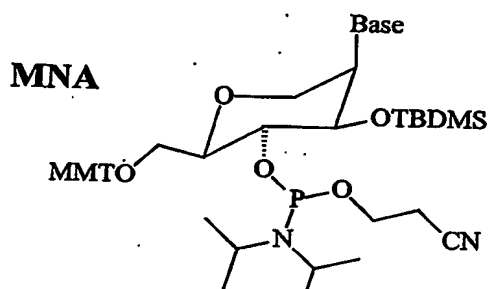
Ref. Nielsen, P. E. et al. *Science*, 1991, 254, 1497.



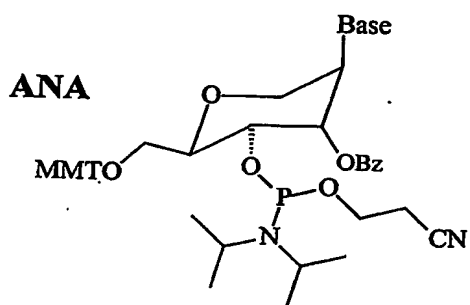
Examples of oligomers of some analogues



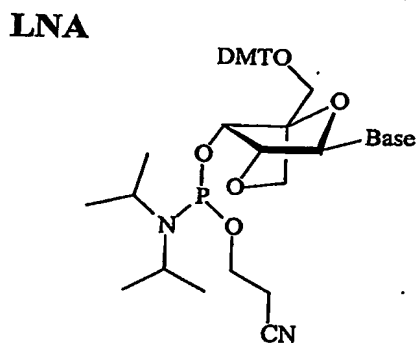
Ref. Van Aerschot, A. et al. *Angew. Chem. Int. Ed. Engl.*, 1995, 34, 1338-1339.



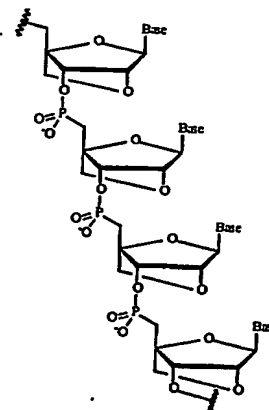
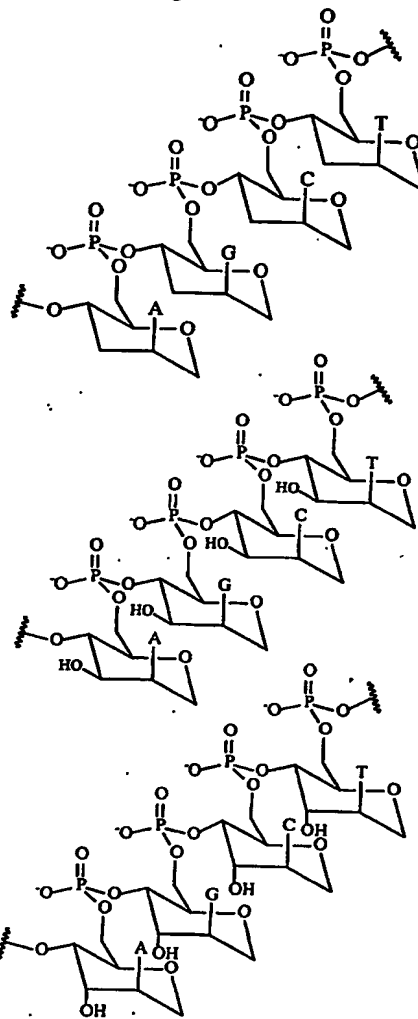
Ref. Hossain N. et al. *J. Org. Chem.*, 1998, 63, 1574-1582.



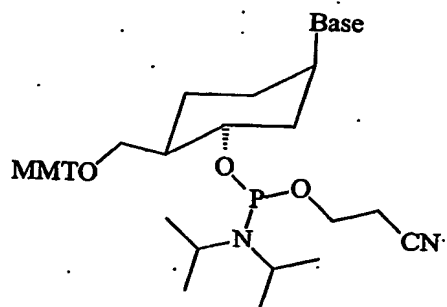
Ref. Allart, B. et al. *Chem. Eur. J.*, 1999, 5, 2424-2431.



Ref. Singh, S. K. et al. *Chem. Commun.*, 1998, 455-456; Koshkin, A. A. et al. *Tetrahedron*, 1998, 54, 3607-3630; Obika, S. et al. *Tetrahedron Lett.*, 1997, 38, 8735-8738.

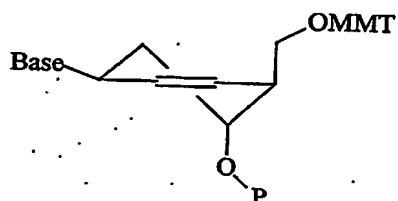


Cyclohexanyl-NA (CNA)



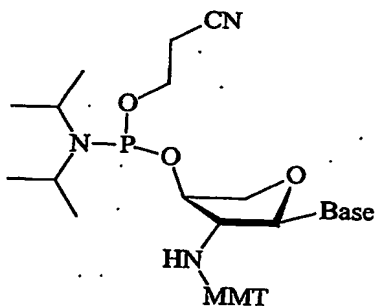
Ref: Maurinsh, Y.; et al. Chem. Eur. J., 1999, 2139-2150.

Cyclohexenyl-NA (CeNA)

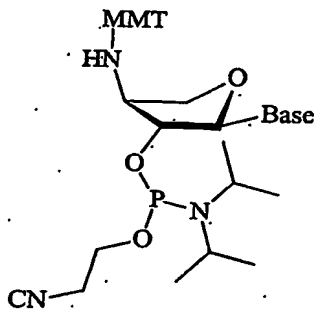


Ref: Wang, J.; et al. J. Am. Chem. Soc, 2000, 8595-8602.

(2'-NH)-TNA

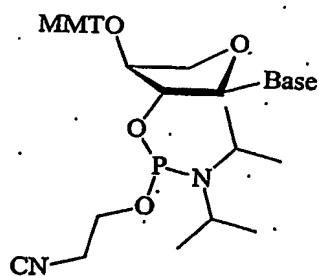


(3'-NH)-TNA



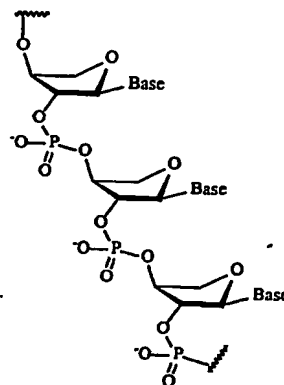
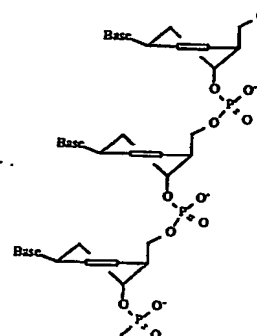
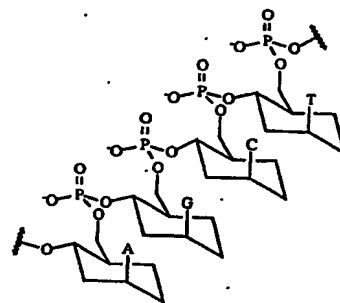
Ref.:Wu, X. et al., Org. Lett., 2002, 4, 1279-1282

TNA

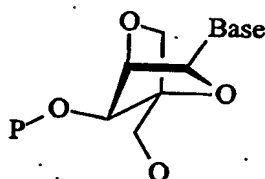


Ref.:Wu, X. et al., Org. Lett., 2002, 4, 1279-1282

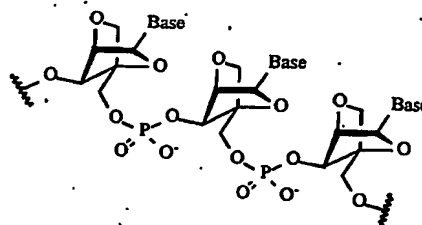
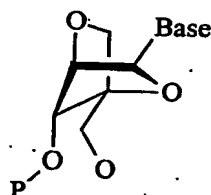
Examples of oligomers of some analogues



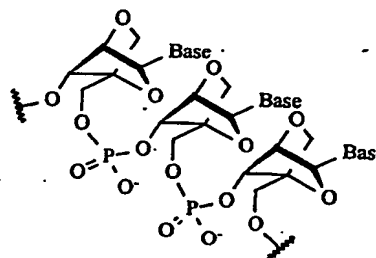
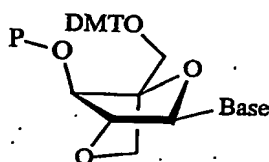
Section of a nucleic acid of the respective analogues

 α -L-Ribo-LNA

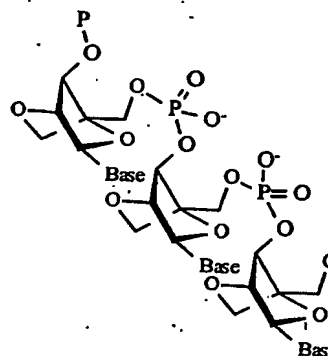
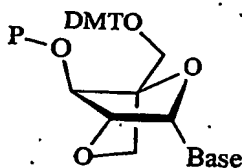
Ref: Rajwanshi, V. K. et al. Chem. Commun., 1999, 1395-1396.

 α -L-Xylo-LNA

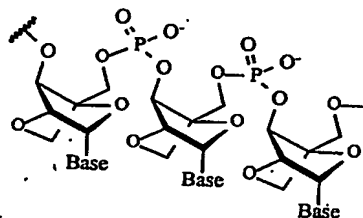
Ref: Rajwanshi, V. K. et al. Angew. Chem. Int. Ed., 2000, 1656-1659.

 β -D-Xylo-LNA

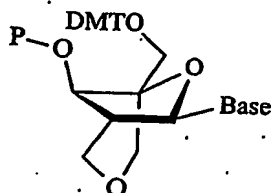
Ref: Rajwanshi, V. K. et al. Chem. Commun., 1999, 1395-1396.

 α -D-Ribo-LNA

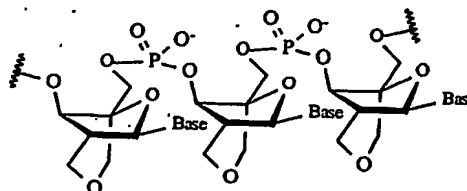
Ref: Rajwanshi, V. K. et al. Angew. Chem. Int. Ed., 2000, 1656-1659.



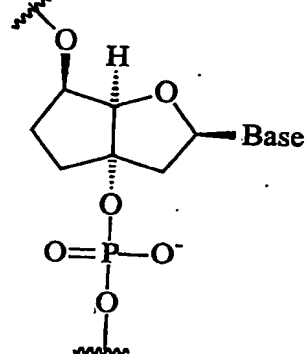
[3.2.1]-LNA



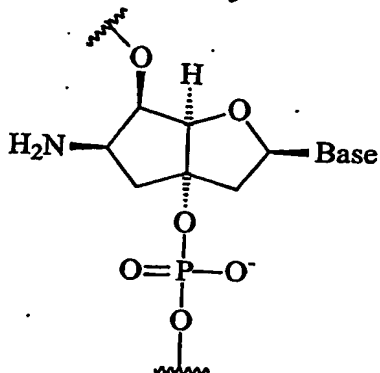
Ref: Wang, G.; et al. Tetrahedron, 1999, 7707-2724.



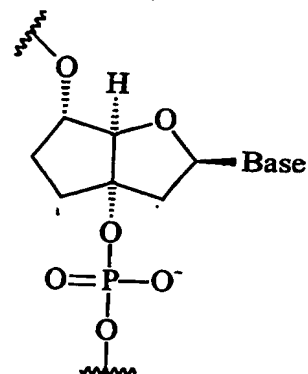
Bicyclo-DNA



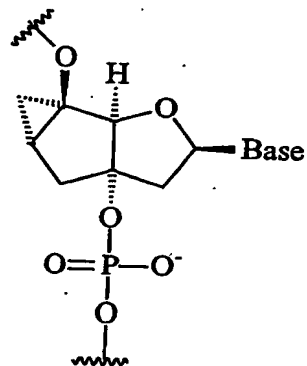
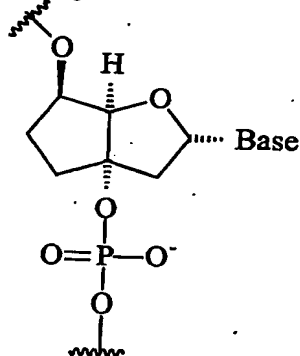
6'-Amino-Bicyclo-DNA



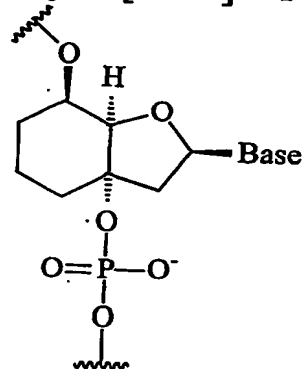
5'-epi-Bicyclo-DNA



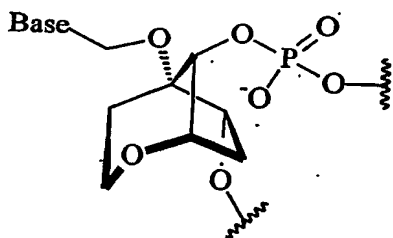
Tricyclo-DNA

 α -Bicyclo-DNA

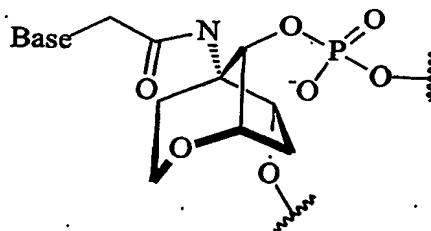
Bicyclo[4.3.0]-DNA



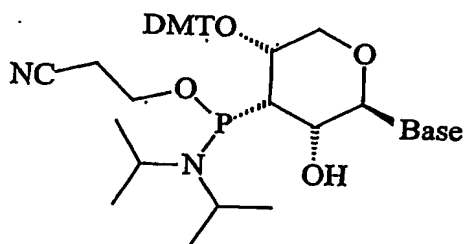
Bicyclo[3.2.1]-DNA



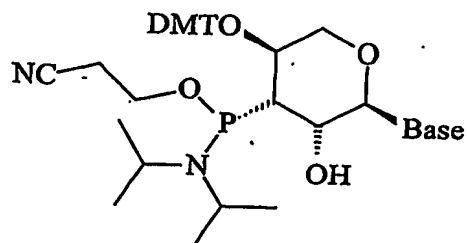
Bicyclo[3.2.1]amide-DNA



Ref: All of the Bicyclo-DNAs are reviewed in Leumann, C. J., Bioorg. Med. Chem., 2002, 841-854.

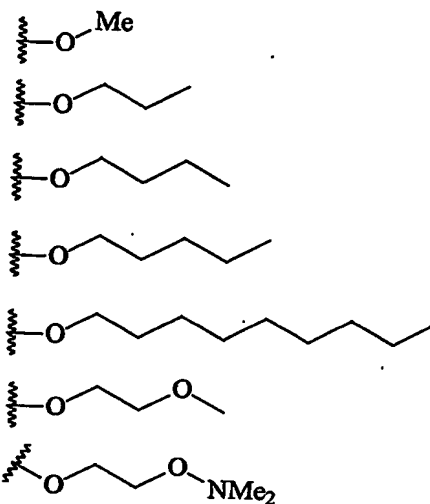
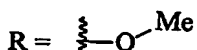
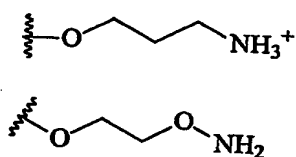
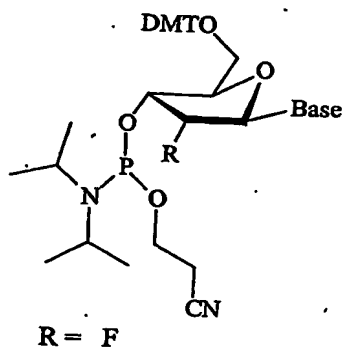
 β -D-Ribopyranosyl-NA

Ref: Reck, F. et al., Org. Lett. 1999, 1, 1531

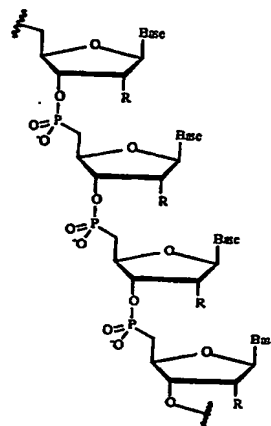
 α -L-Lyxopyranosyl-NA

Ref: Reck, F. et al., Org. Lett. 1999, 1, 1531

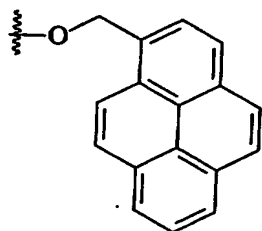
2'-R-RNA



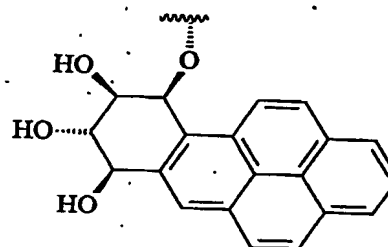
General structure of 2'-modified oligomers



Ref: Reviewed by Manoharan, M. Biochim. BioPhys. Acta, 1999, 117-130.



Ref: Yamana, K. et al., Tetrahedron Lett., 1991, 6347-6350.



Ref: Sayer, J. et al., J. Org. Chem., 1991, 56, 20-29.

Examples of modifications that, to our knowledge, are not synthesised or published yet:

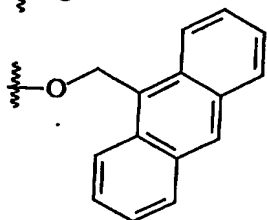
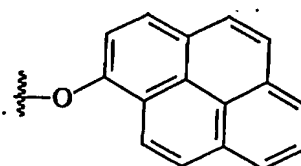
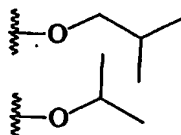
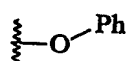
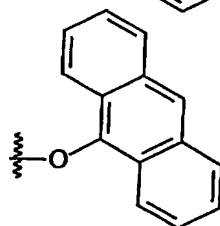
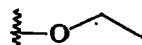
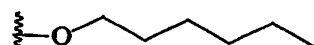
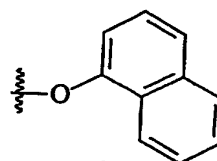
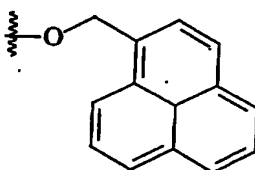
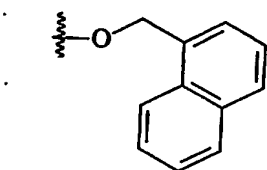
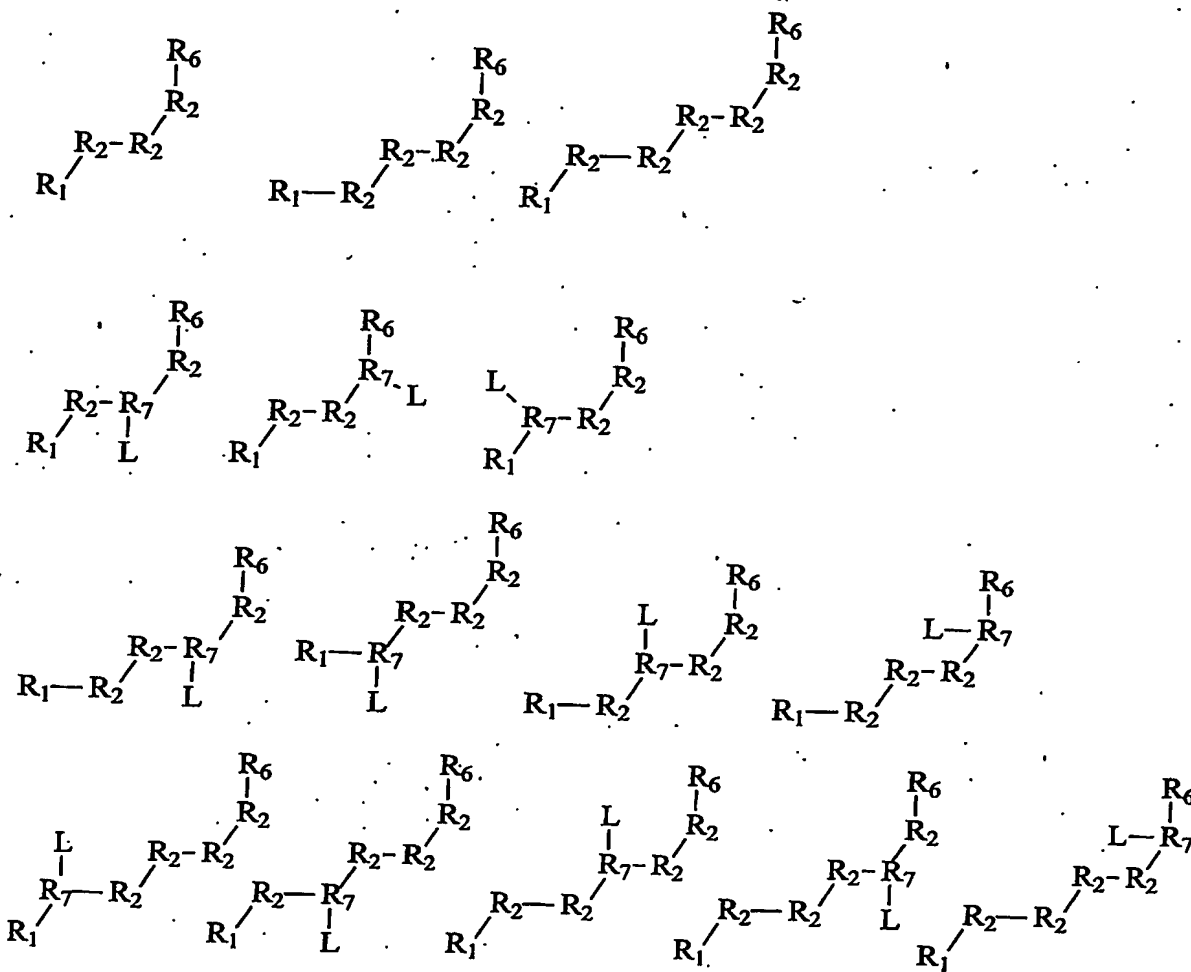
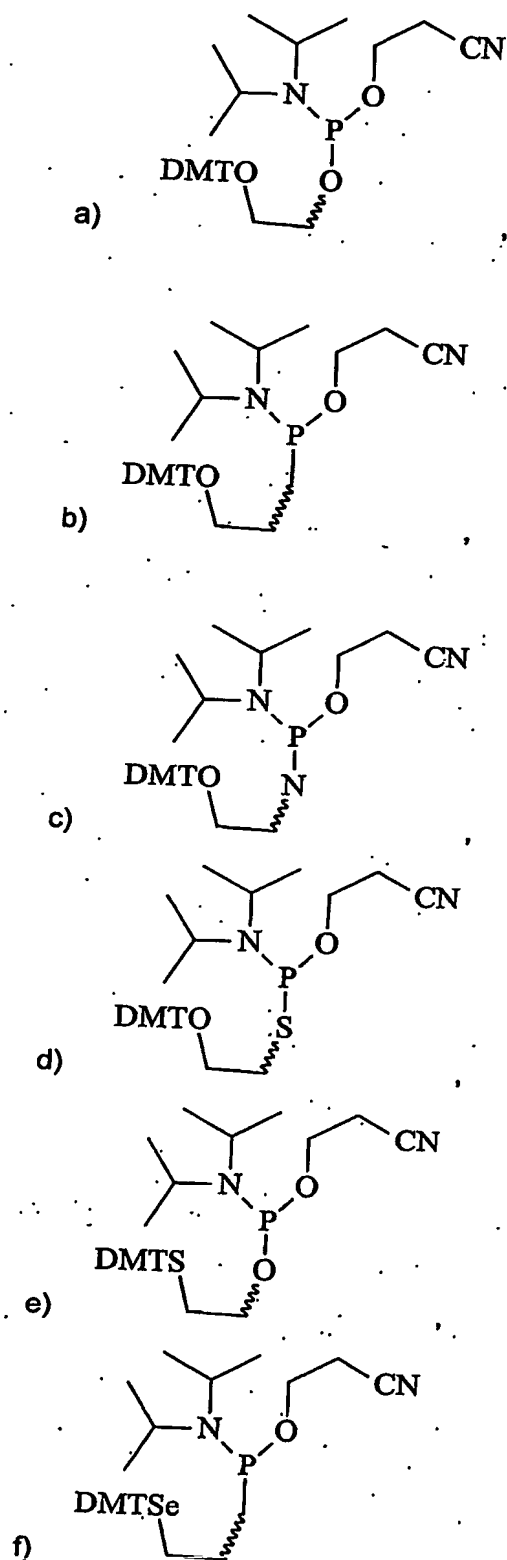


Figure 2.



wherein R_1 , R_2 and R_6 are as defined in the in the accompanying description, and $R_7 =$ N, or CH.

Figure 3. Acyclic backbone monomer units.



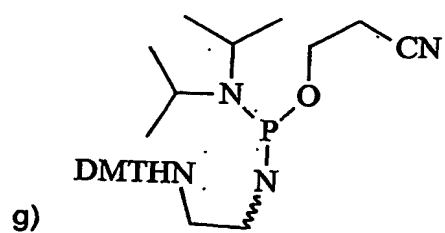
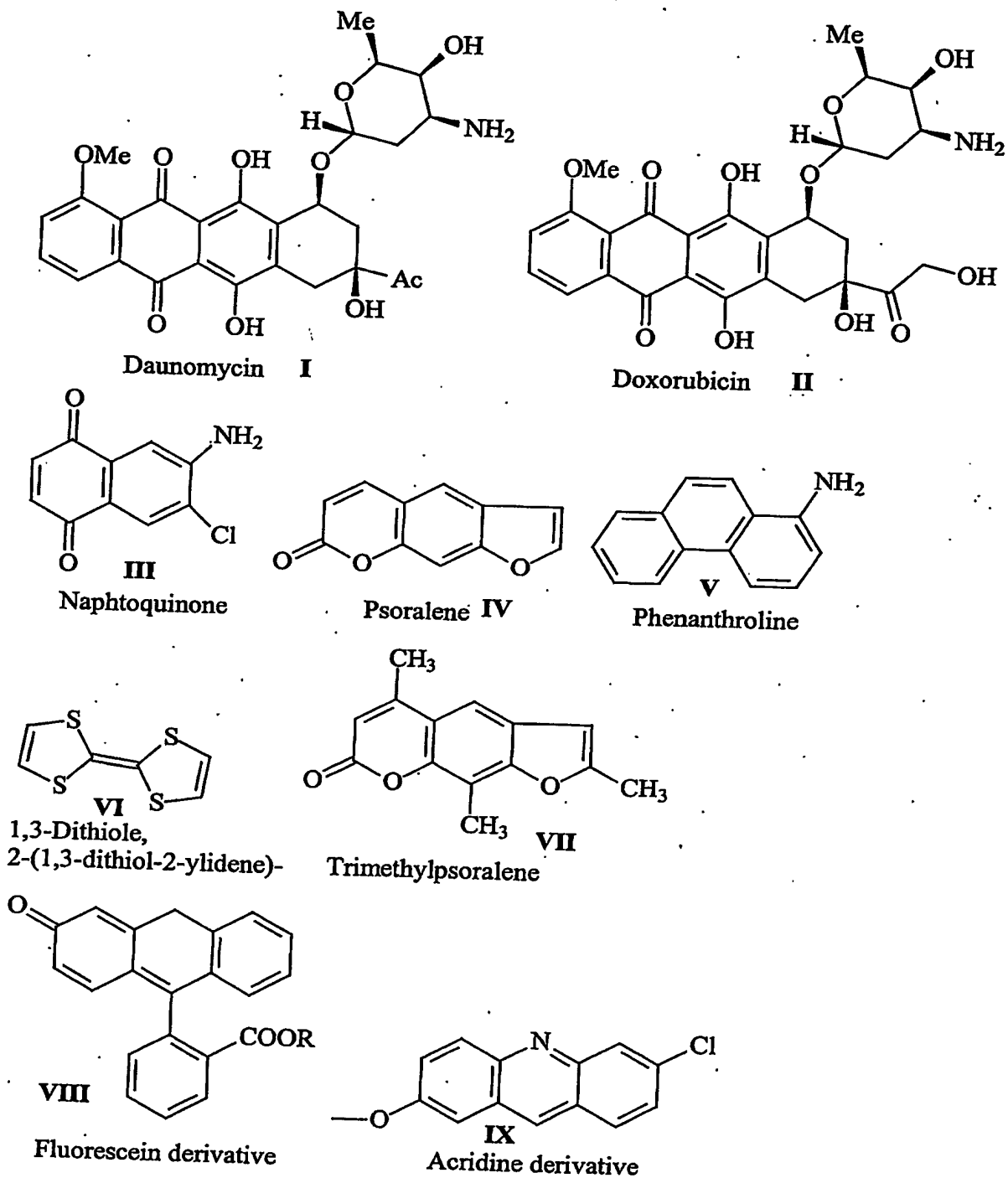
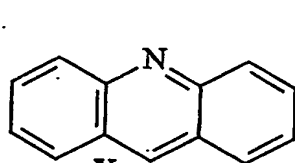
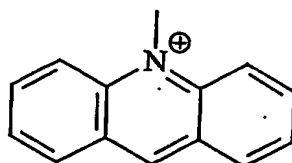


Figure 4. Preferred intercalators.

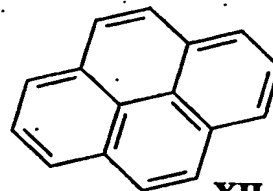




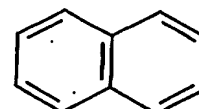
X
Acradine



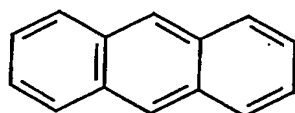
XI
Acradinium



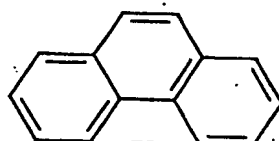
XII
Pyrene



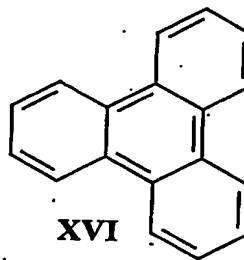
XIII
Naphthalene



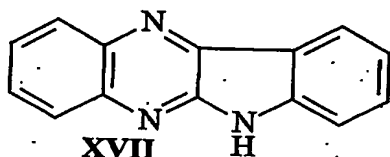
XIV
Anthracene



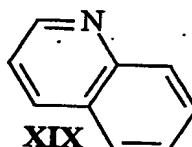
XV
Phenanthrene



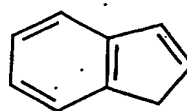
XVI
Triphenylene



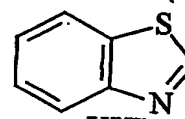
XVII
6H-Indolo[2,3-b]quinoxaline



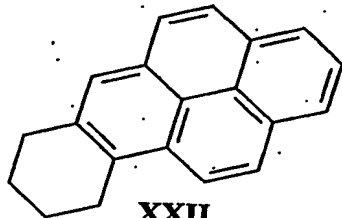
XIX
Quinoline



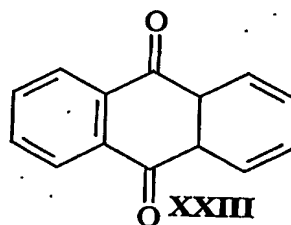
XX
1H-Indene



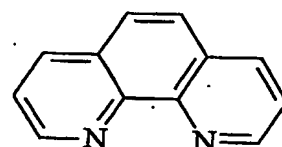
XXI
Benzothiazole



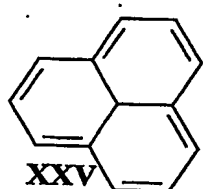
XXII
7,8,9,10-Tetrahydrobenzo[a]pyrene



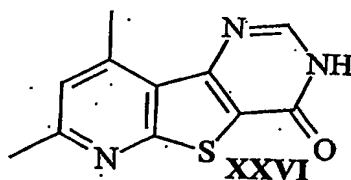
XXIII
9,10-Anthracenedione



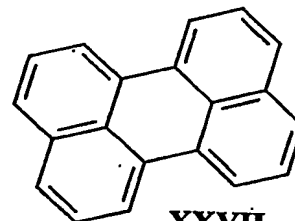
XXIV
1,10-Phenanthroline



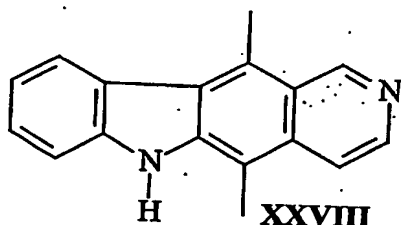
XXV
9bH-Phenalene



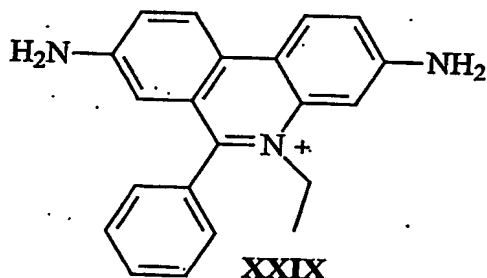
XXVI
Pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-
4(1H)-one, 7,9-dimethyl-



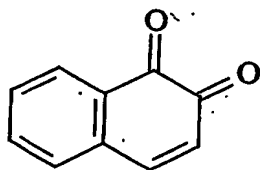
XXVII
Perylene



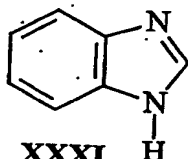
XXVIII
6H-Pyrido[4,3-b]carbazole,
5,11-dimethyl-



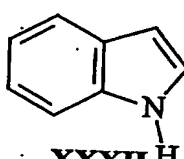
XXIX
Phenanthridinium,
3,8-diamino-5-ethyl-6-phenyl-



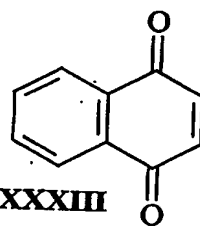
XXX
1,2-Naphthalenedione



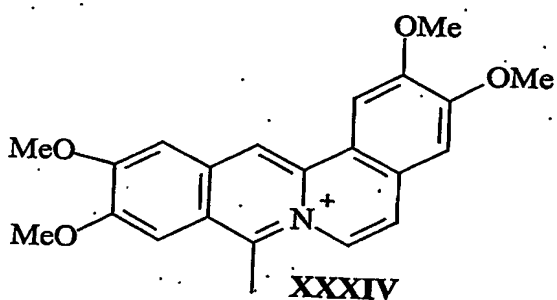
XXXI
1H-Benzimidazole



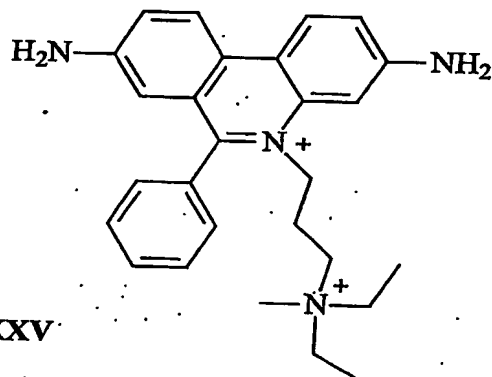
XXXII
1H-Indole



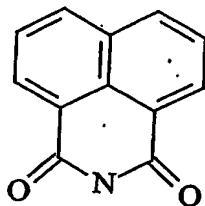
XXXIII
1,4-Naphthalenedione



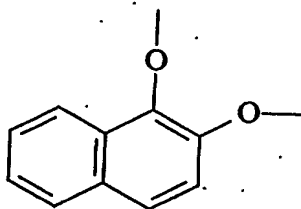
XXXIV
Dibenzo[a,g]quinolizinium,
2,3,10,11-tetramethoxy-8-methyl-



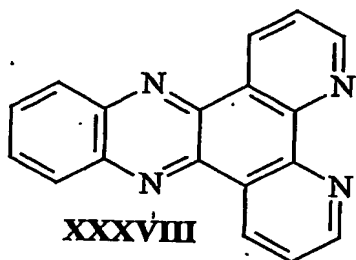
XXXV
Phenanthridinium, 3,8-diamino-5-
[3-(diethylmethylammonio)propyl]-6-phenyl-



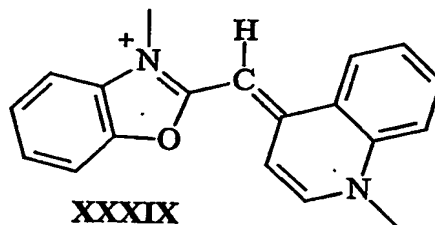
XXXVI
1H-Benz[de]isoquinoline-
1,3(2H)-dione



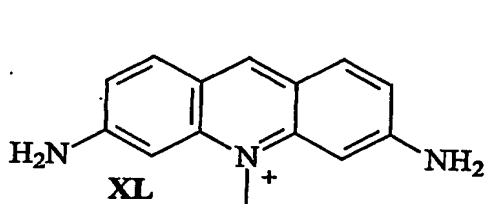
XXXVII
Naphthalene, 1,2-dimethoxy-



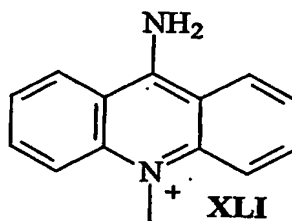
Dipyrido[3,2-a:2',3'-c]phenazine



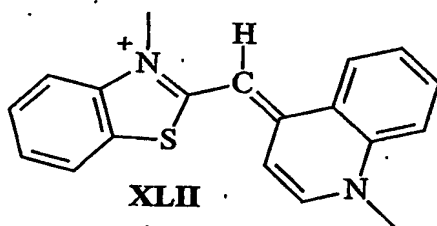
Quinolinium, 4-[(3-ethyl-2(3H)-benzoxazolylidene)methyl]-1-methyl-



Acridine, 6-amino-3,10-dihydro-3-imino-10-methyl-

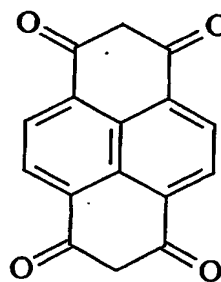


Acridinium, 9-amino-10-methyl-

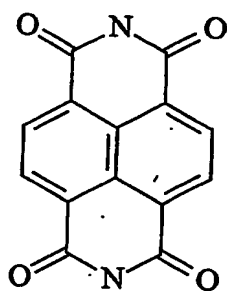


Quinolinium, 1-methyl-4-

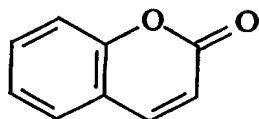
[(3-methyl-2(3H)-benzothiazolylidene)methyl]-



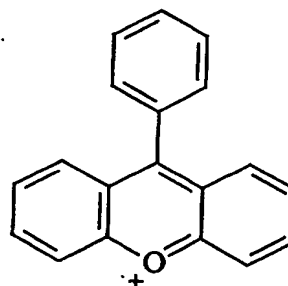
1,3,6,8(2H,7H)-Pyrenetetrone

**XLIV**

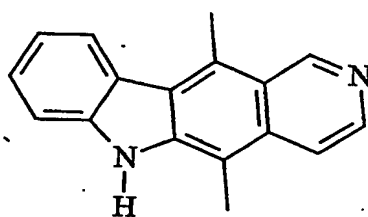
Benzo[lmn][3,8]phenanthroline-
1,3,6,8(2H,7H)-tetrone

**XLV**

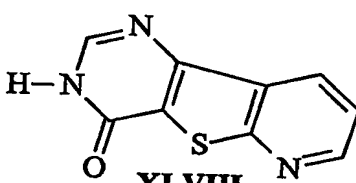
2H-1-Benzopyran-2-one

**XLVI**

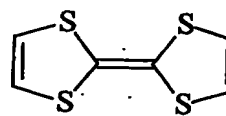
Xanthylium, 9-phenyl-

**XLVII**

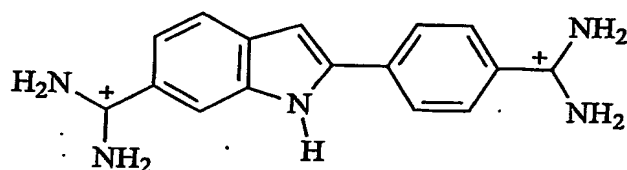
Ellipticine

**XLVIII**

Pyrido[3',2':4,5]thieno
[3,2-d]pyrimidin-
4(1H)-one

**XLIX**

Fulvalene

**L**

DAPI

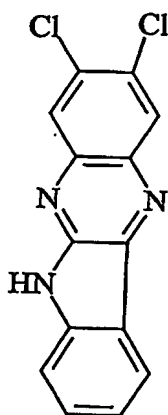
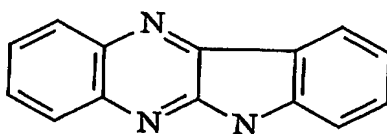
**LI****LII**

Figure 5. Preferred linker chains.

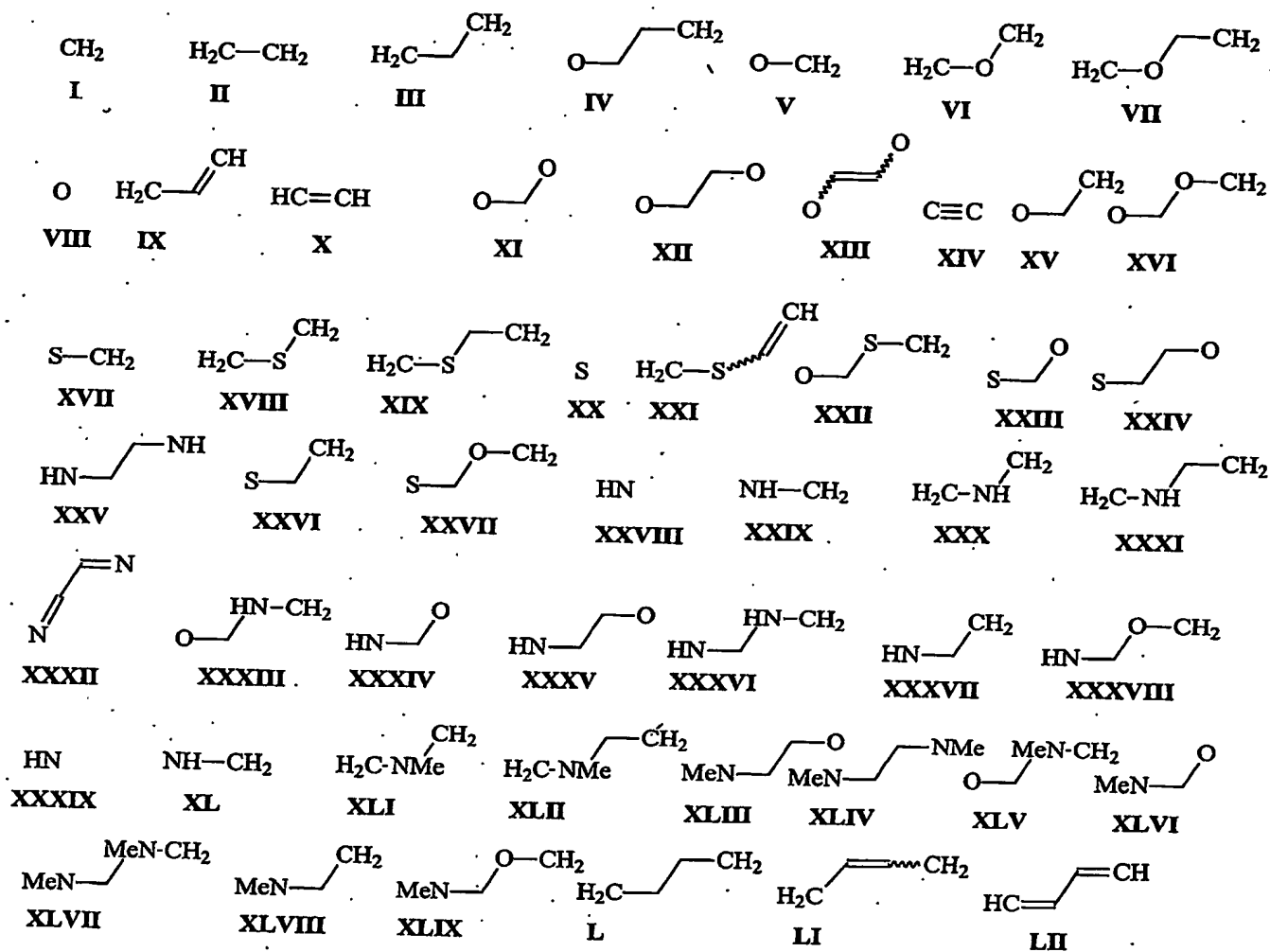
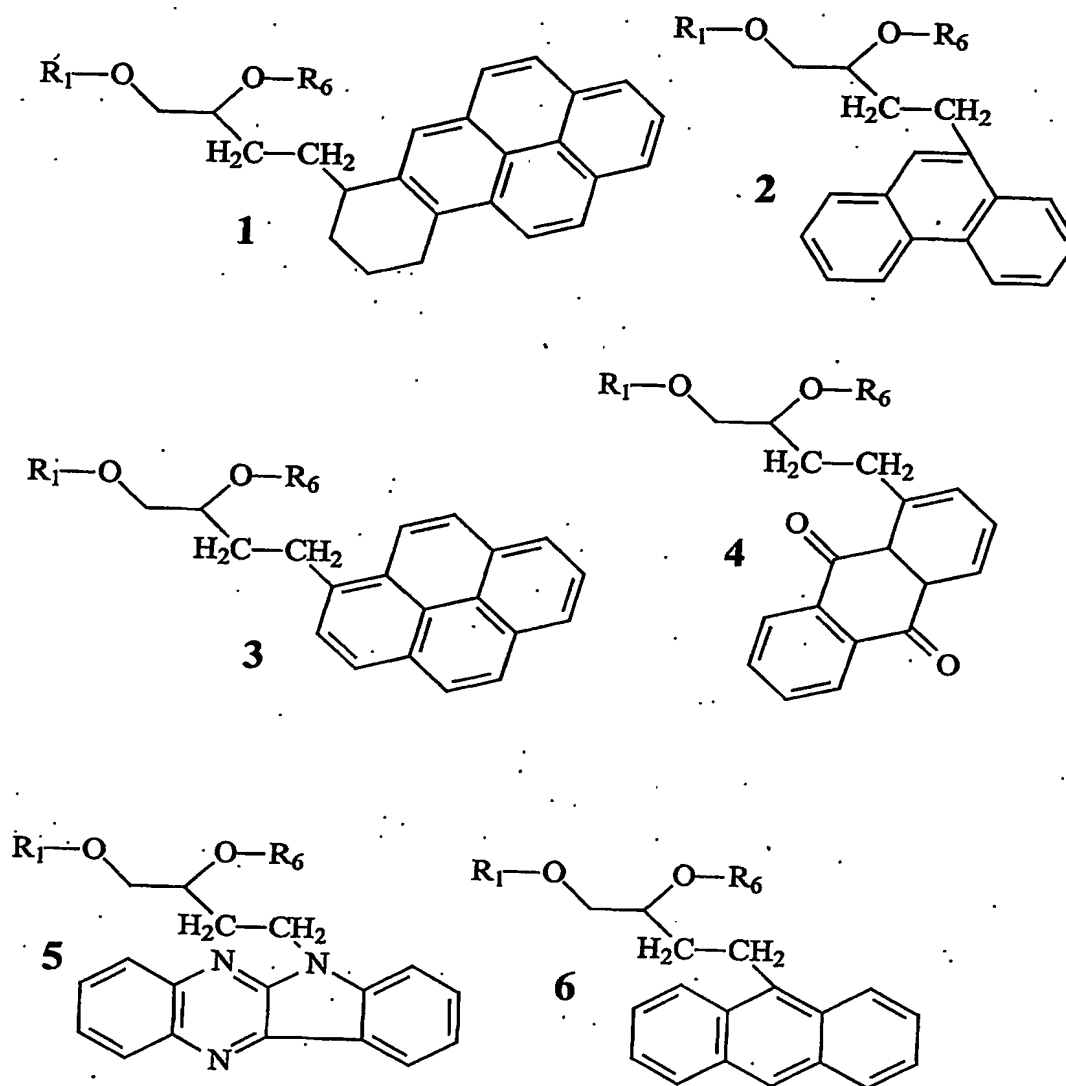
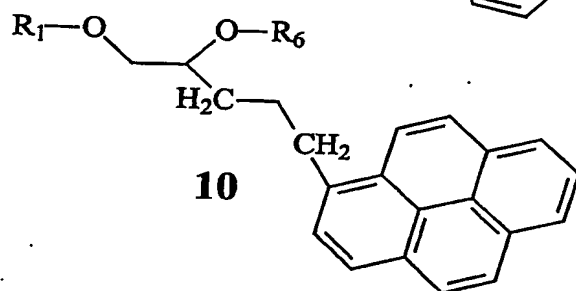
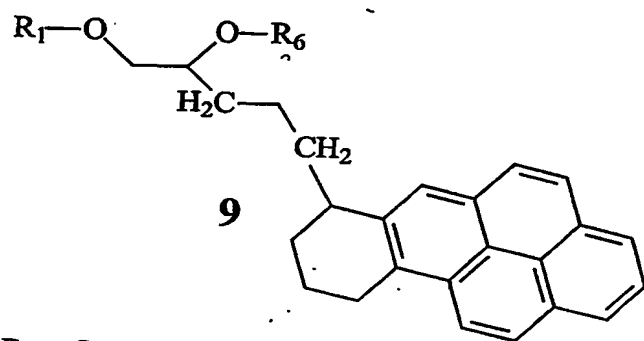
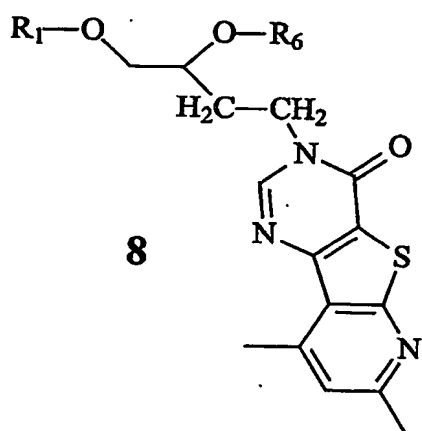
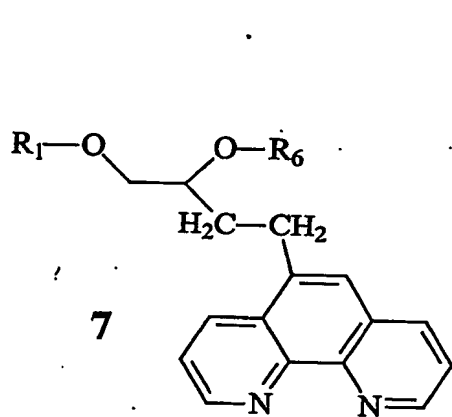
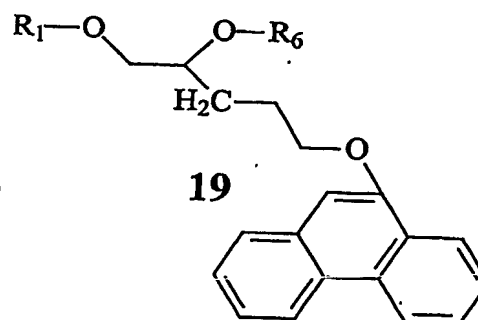
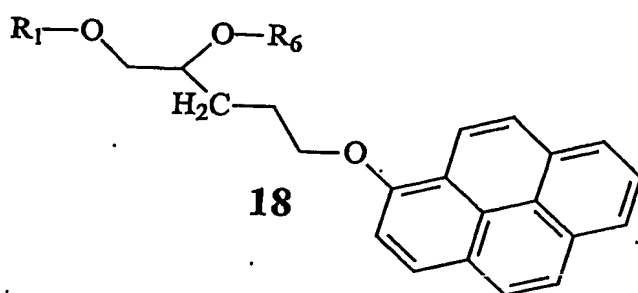
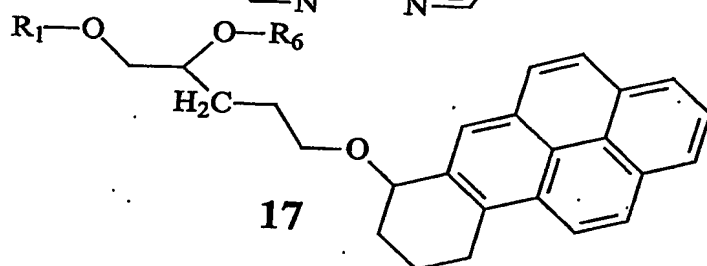
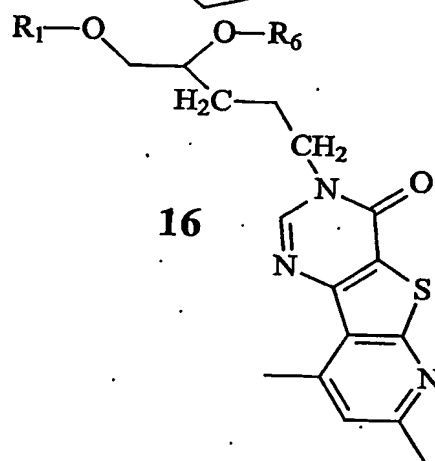
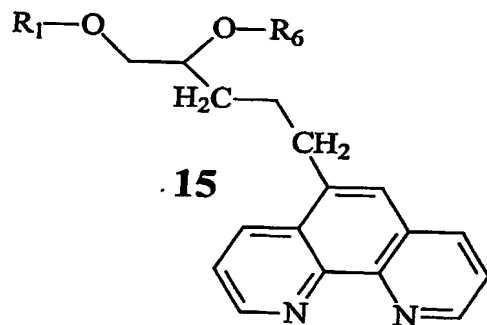
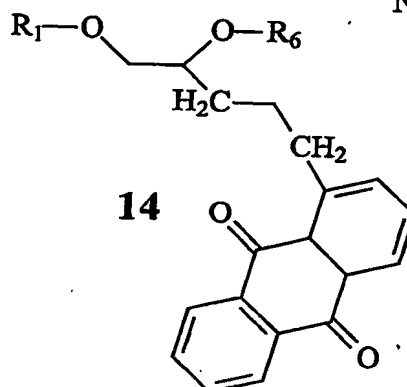
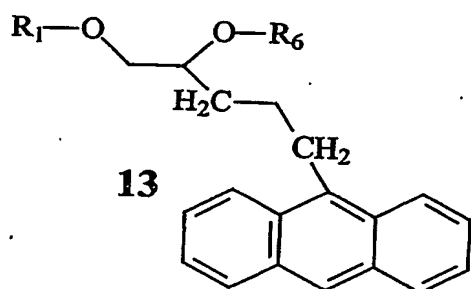
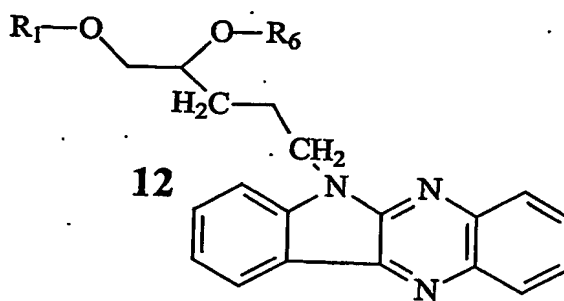
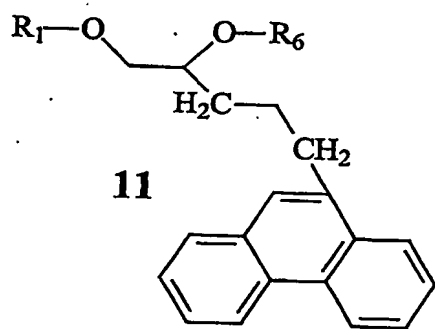
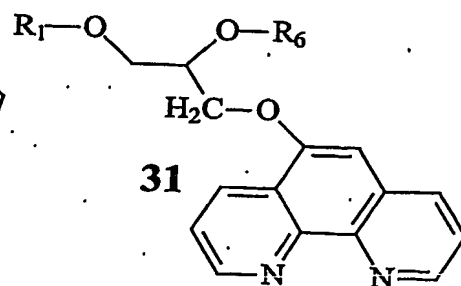
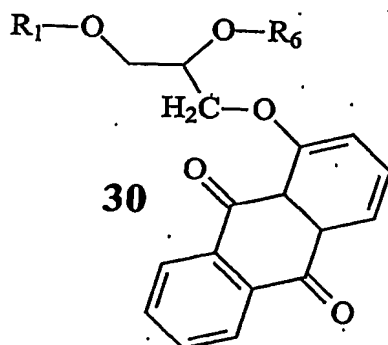
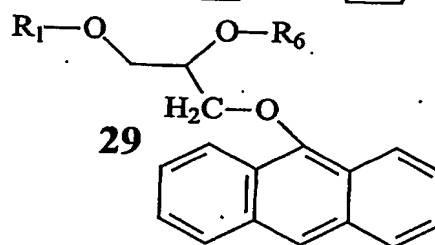
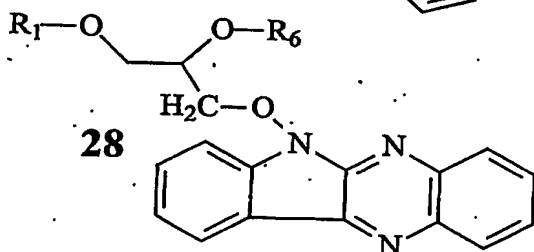
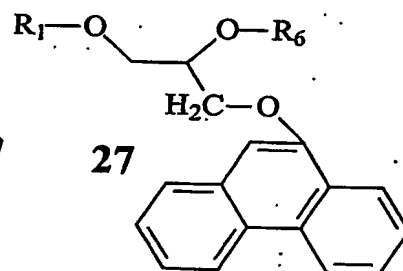
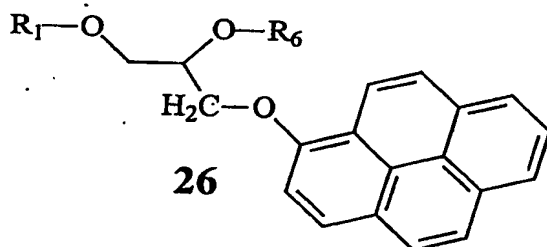
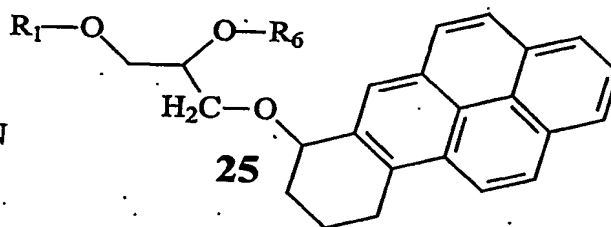
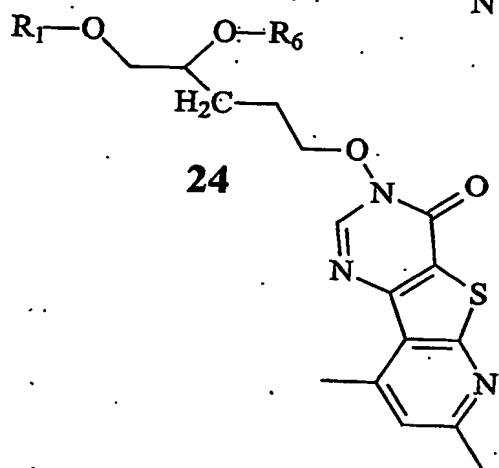
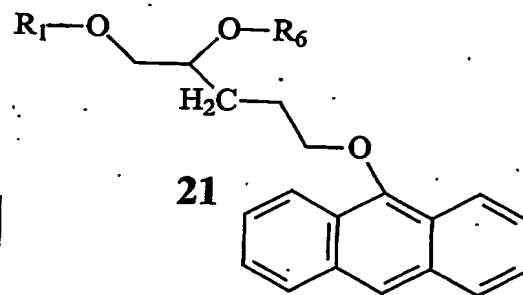
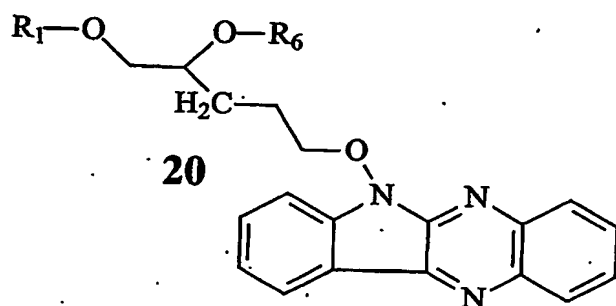


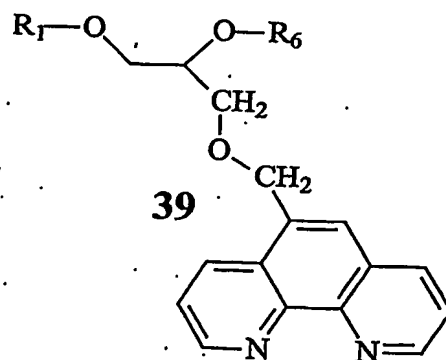
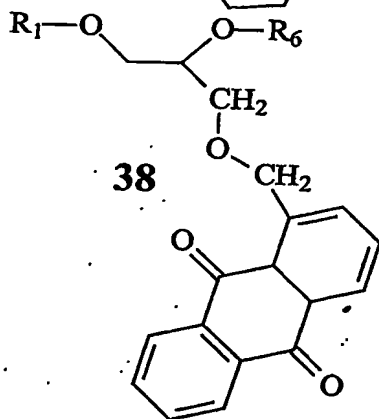
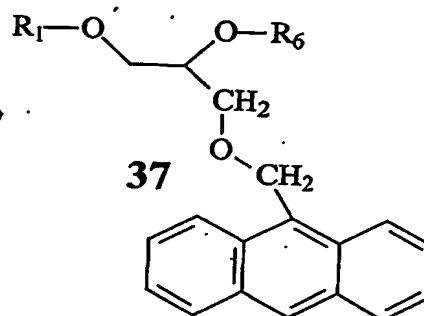
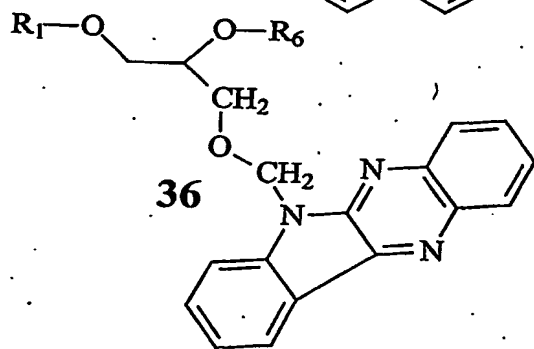
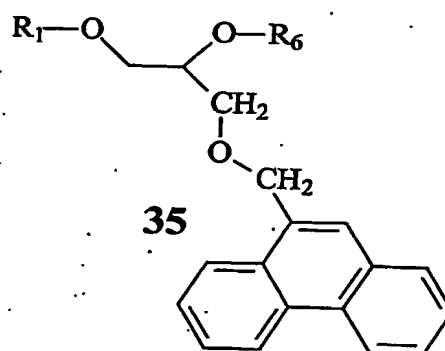
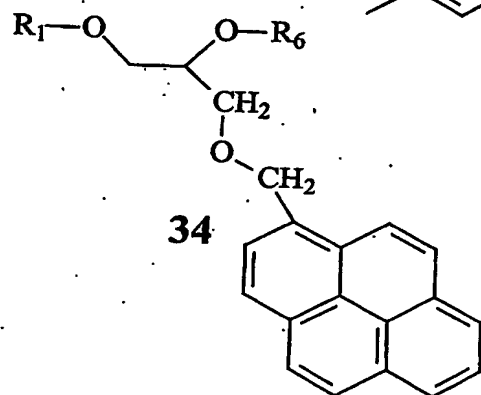
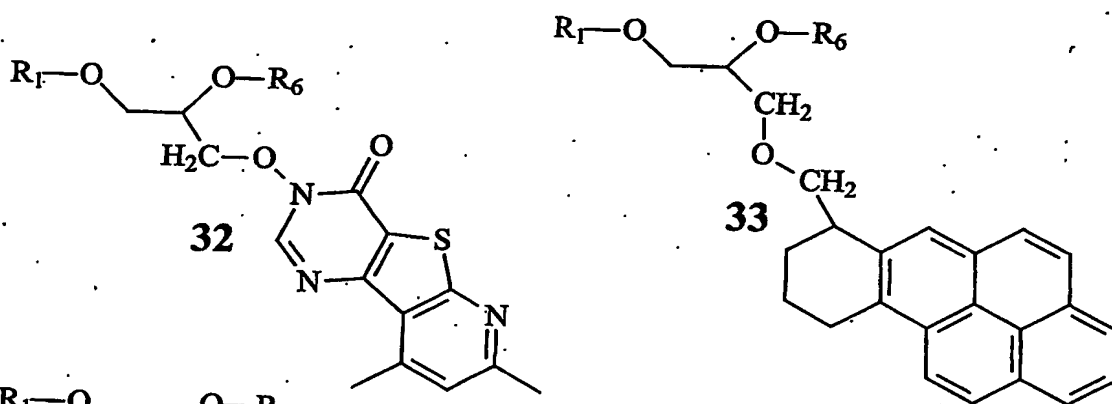
Figure 6. Preferred INA (Intercalator pseudonucleotides).

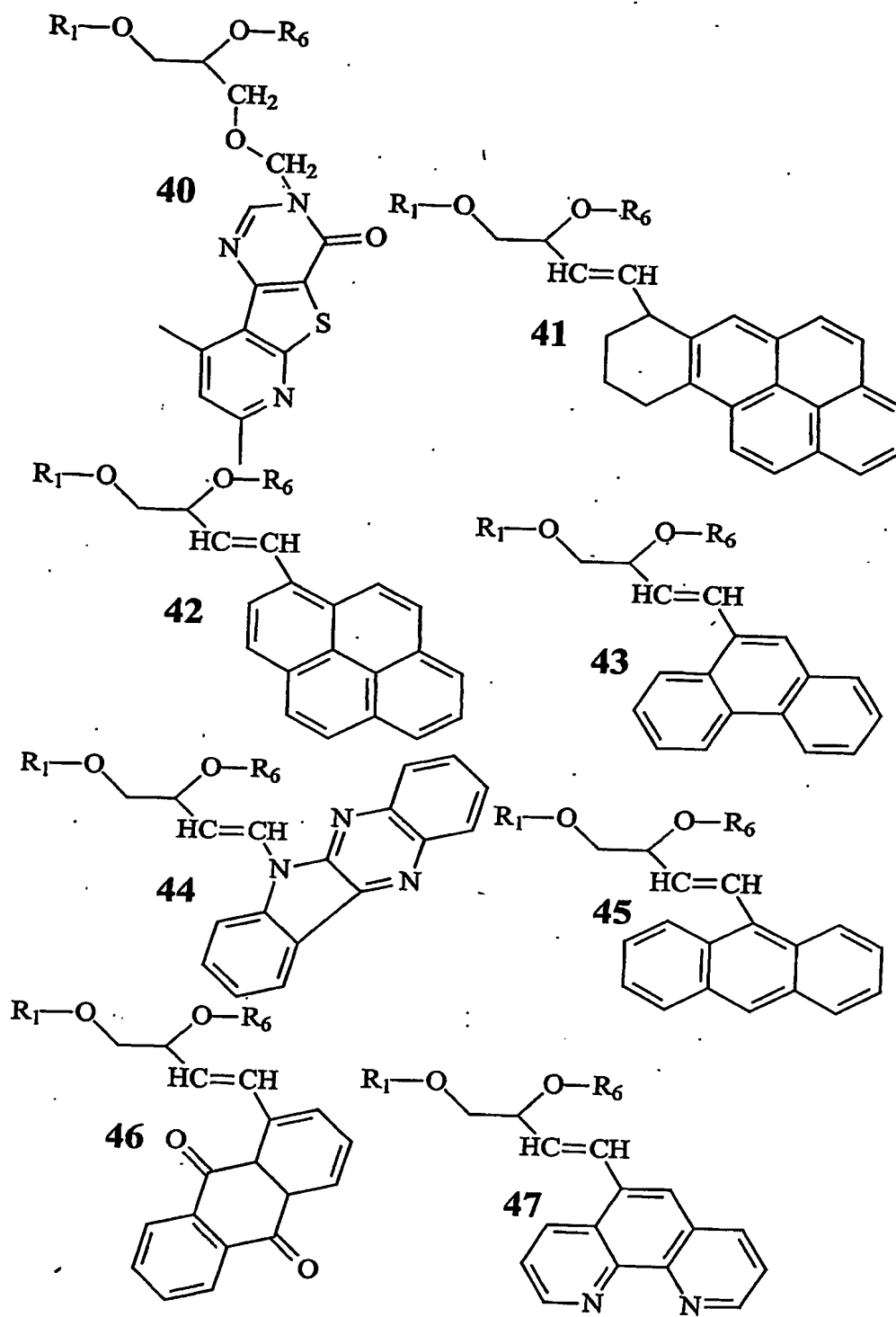


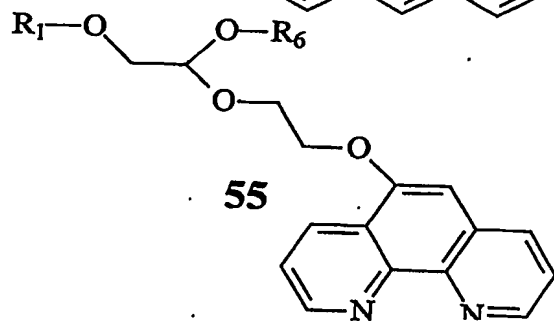
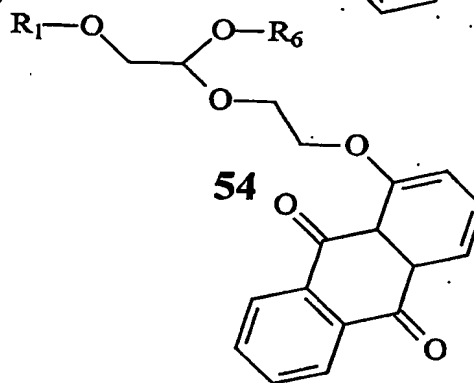
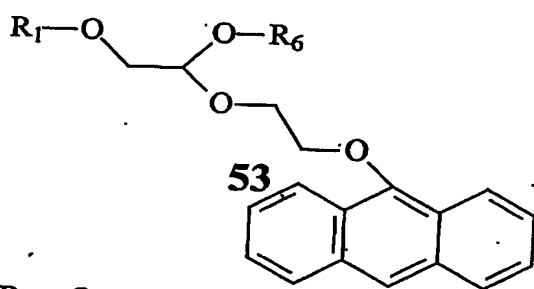
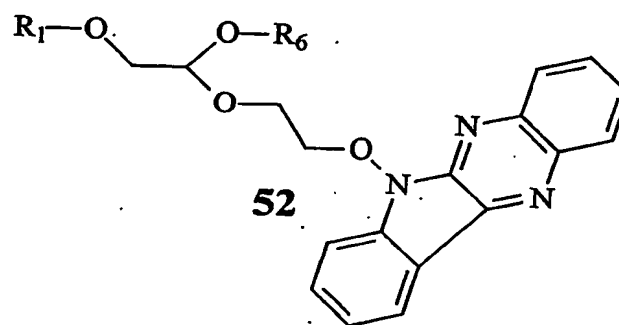
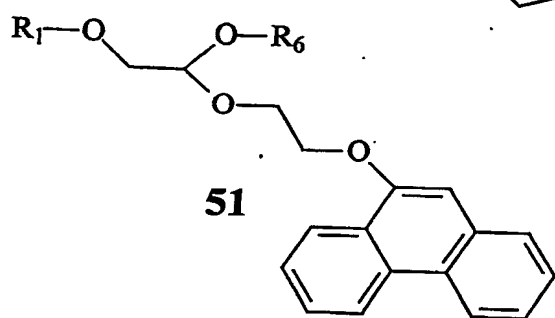
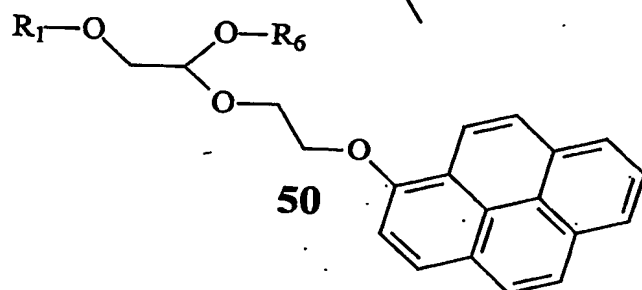
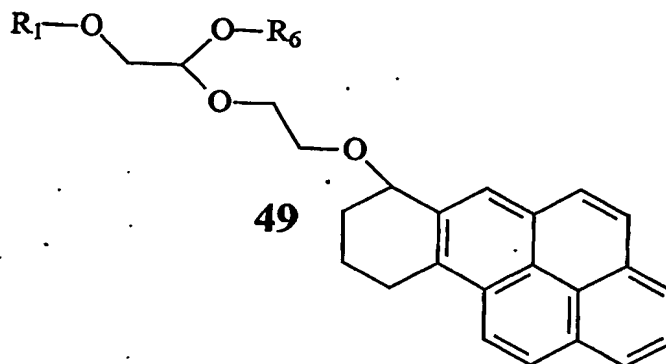
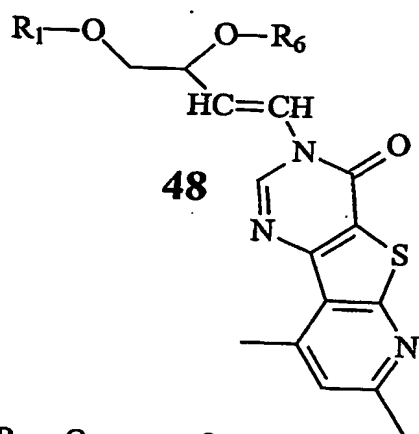


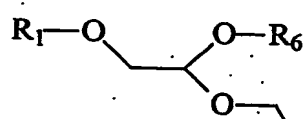
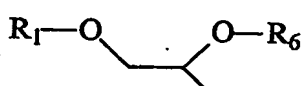
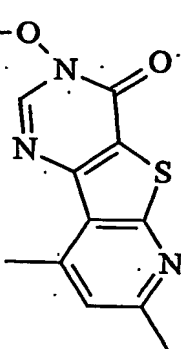
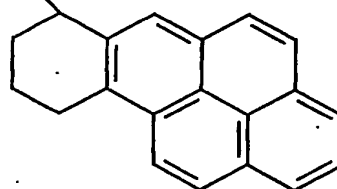
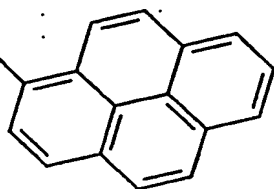
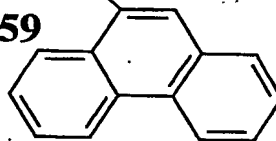
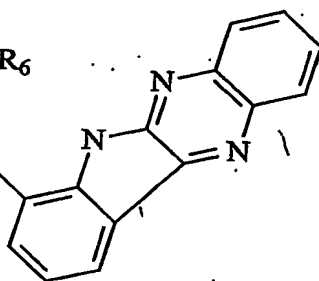
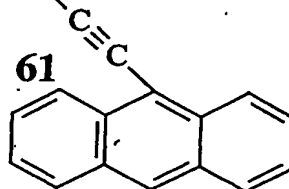


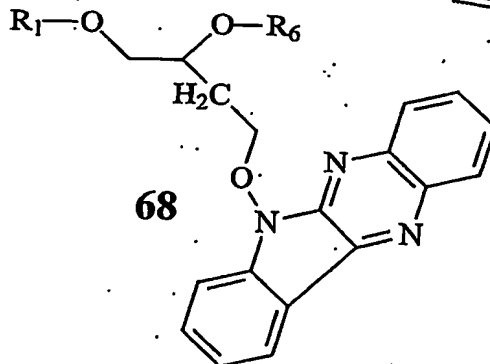
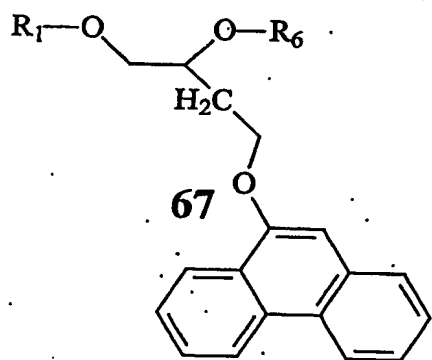
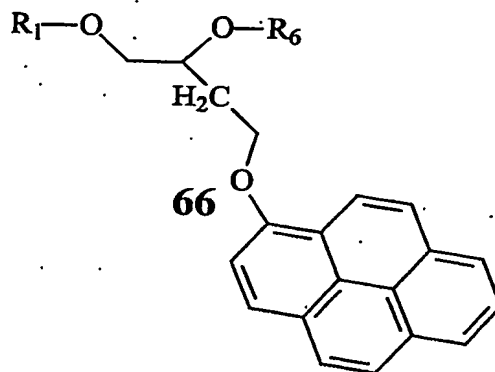
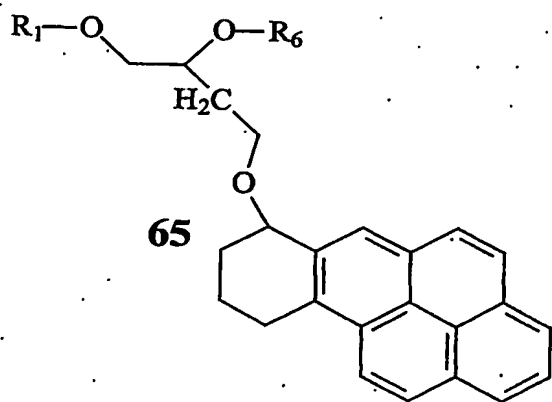
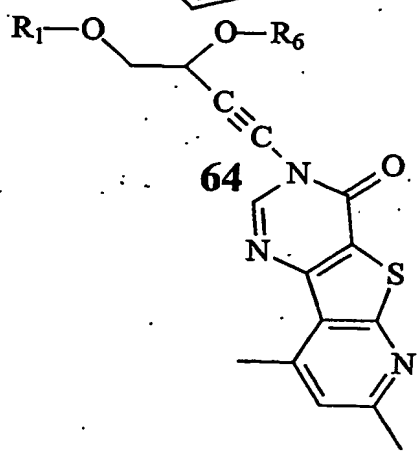
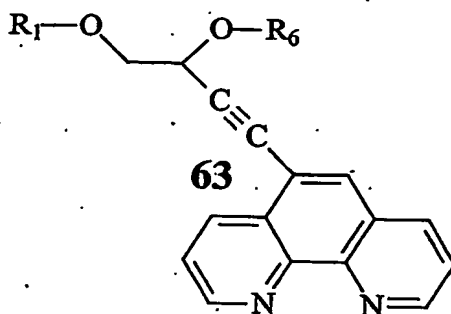
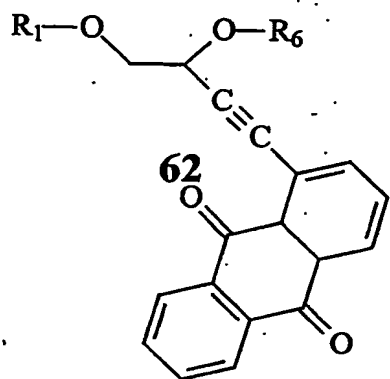


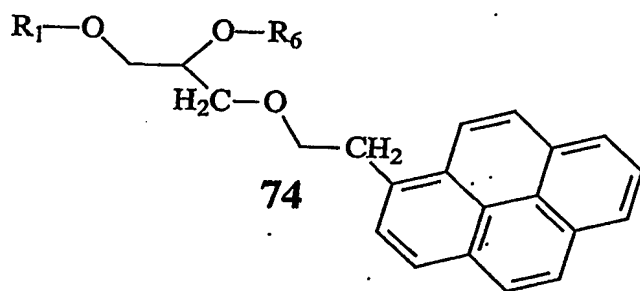
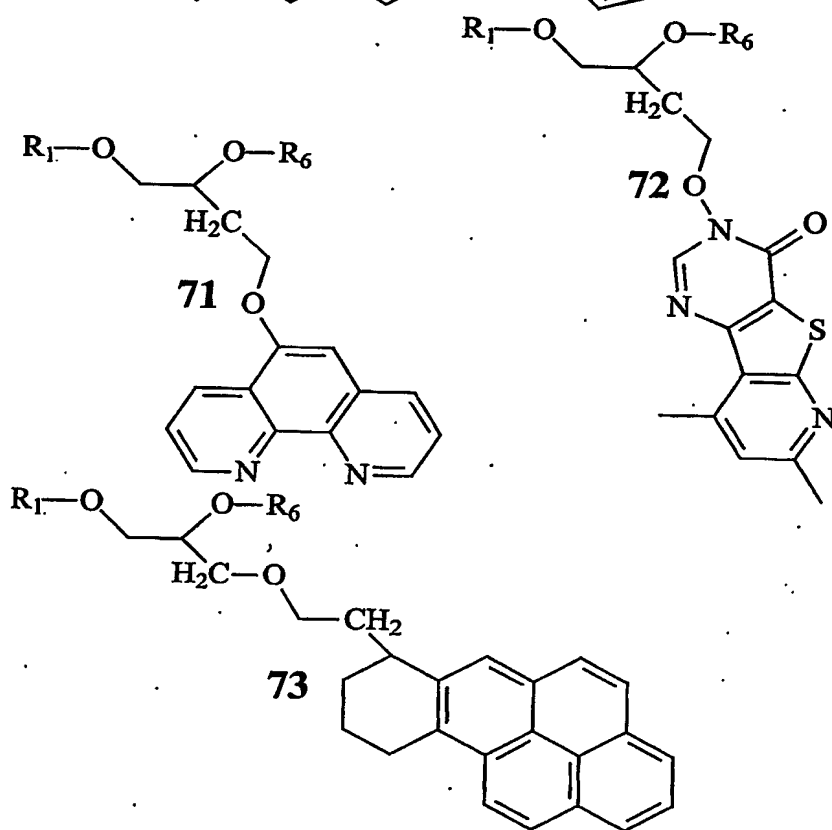
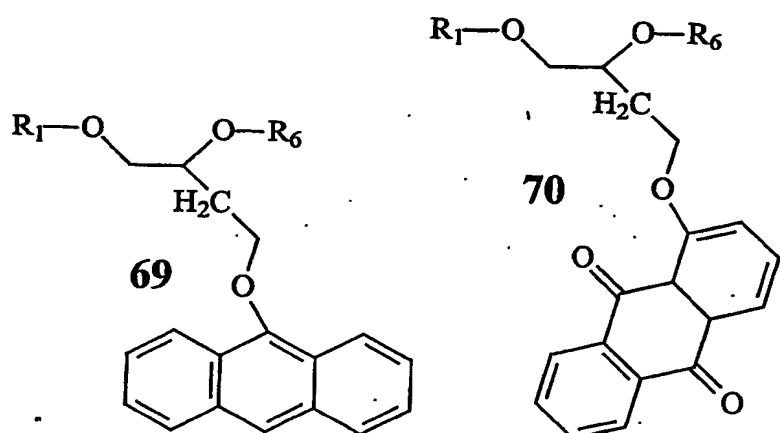


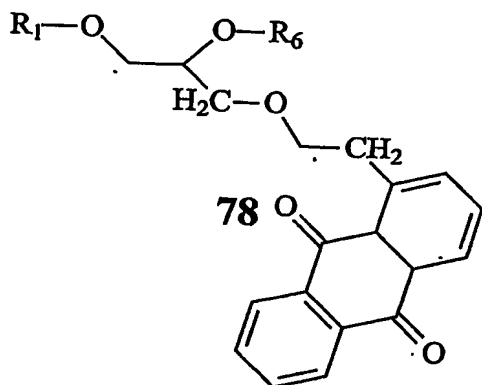
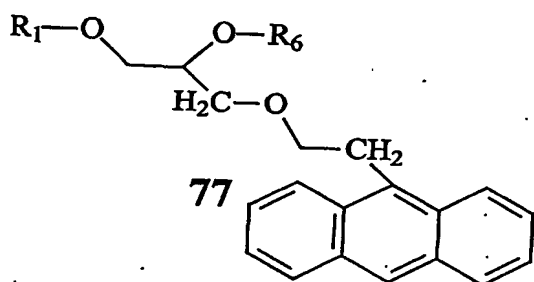
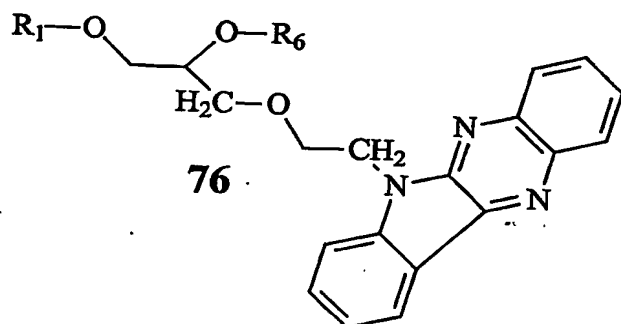
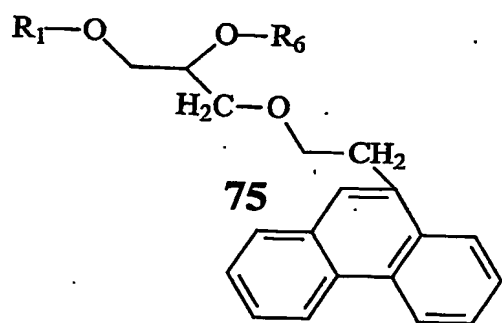


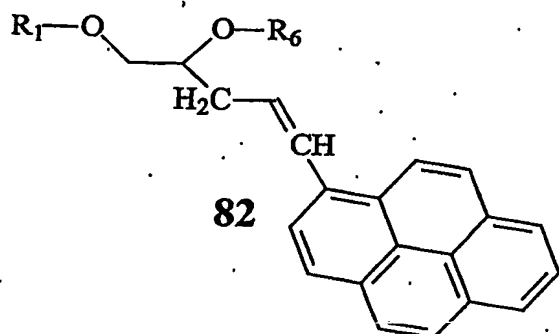
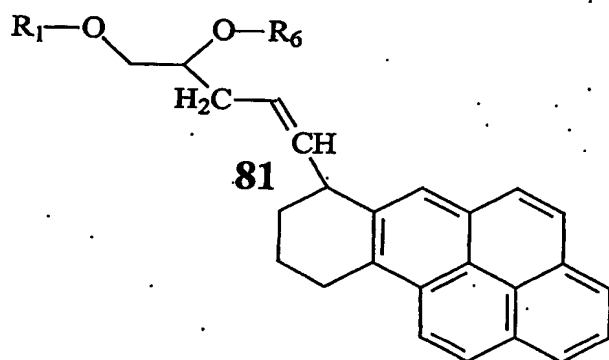
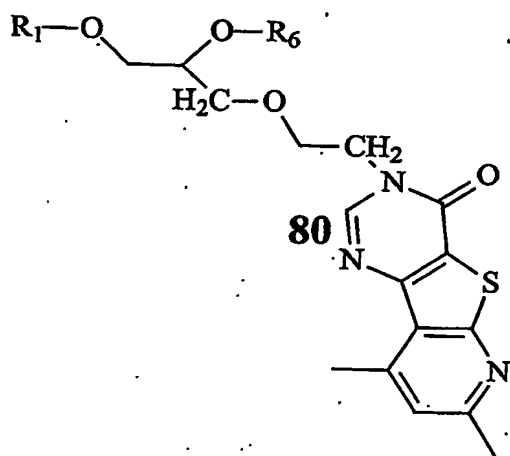
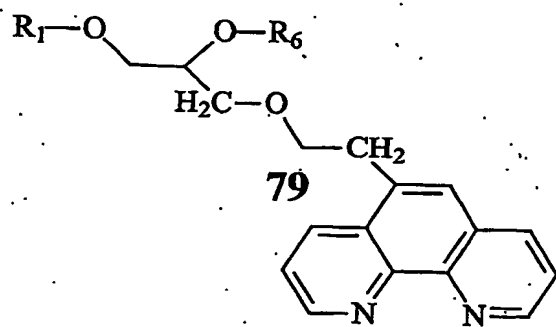


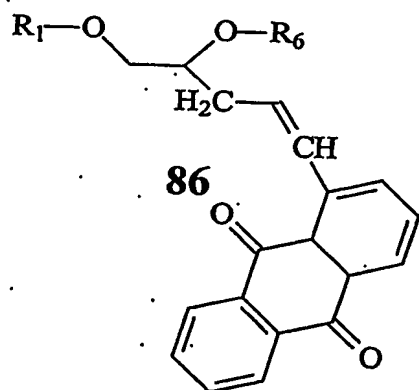
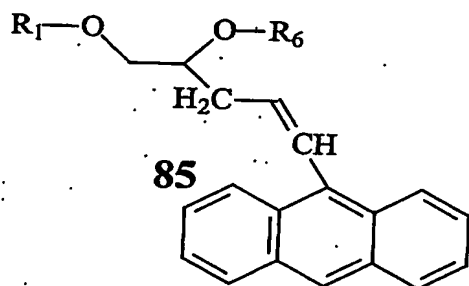
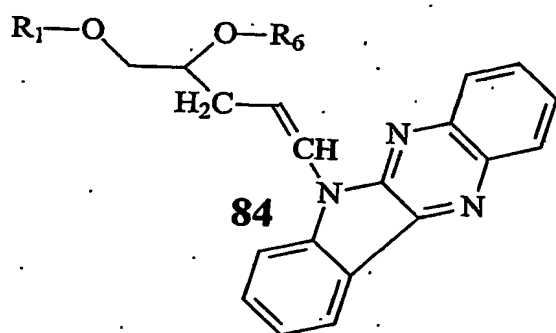
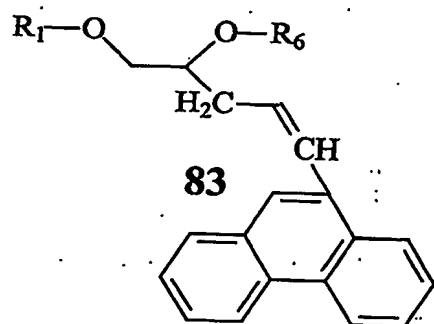
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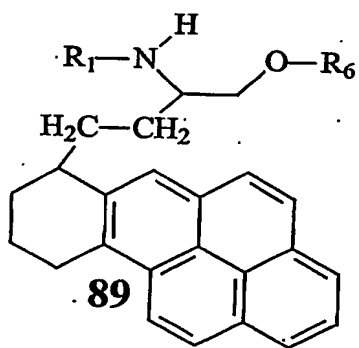
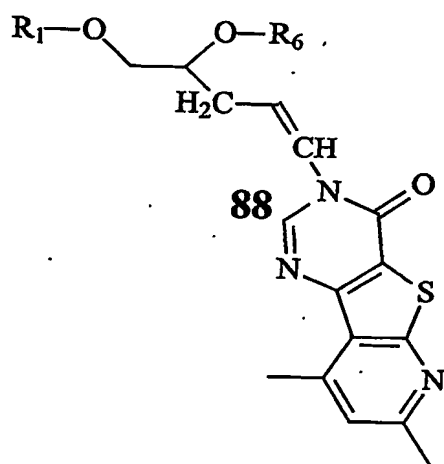
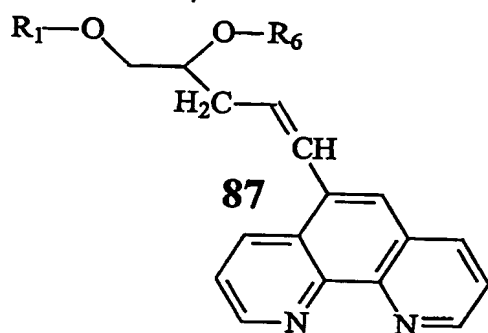


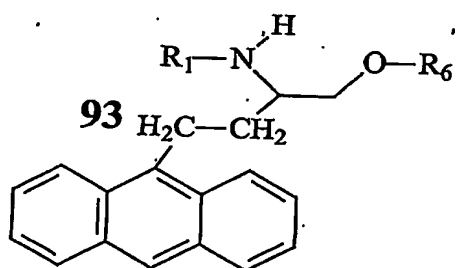
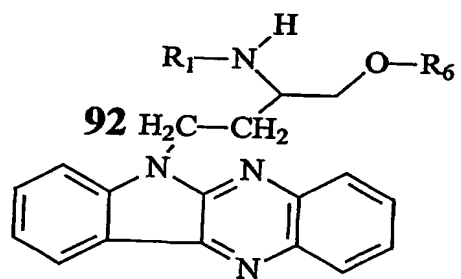
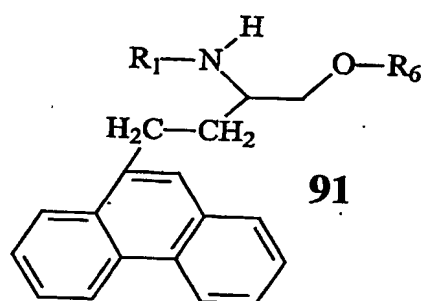
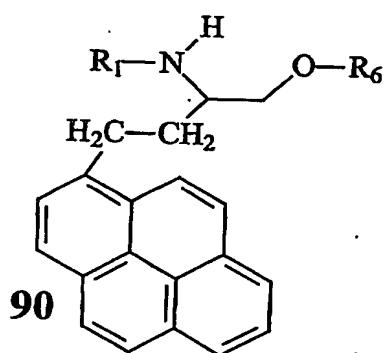


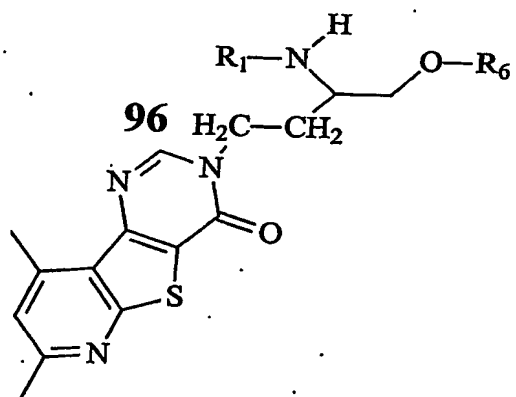
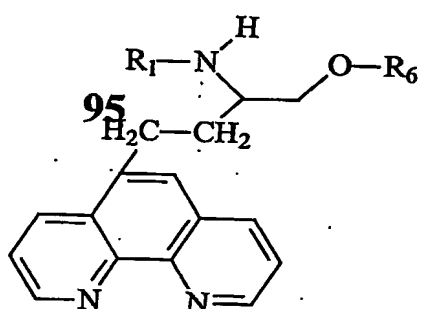
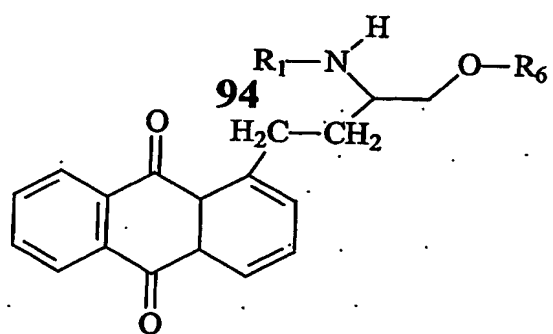


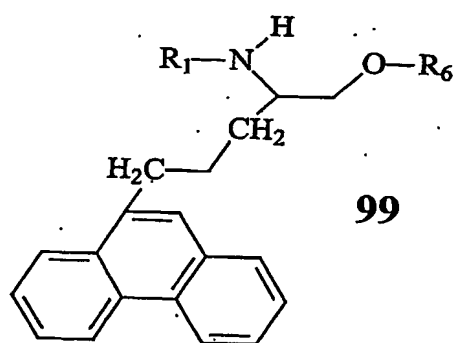
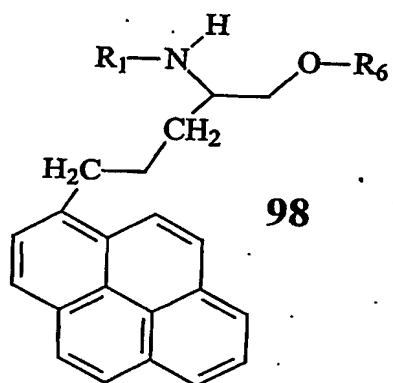
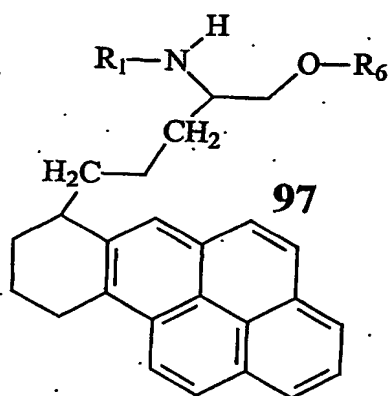


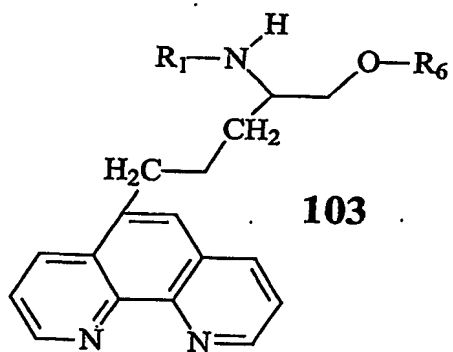
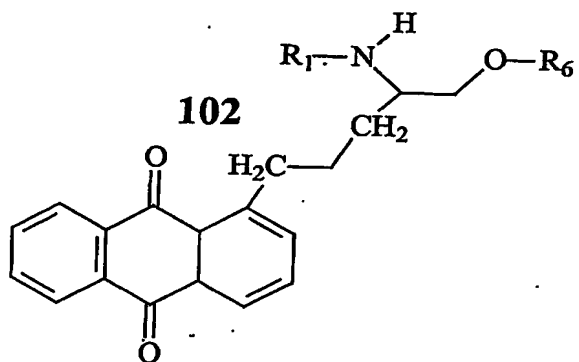
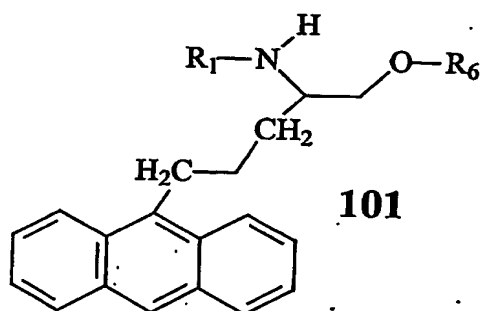
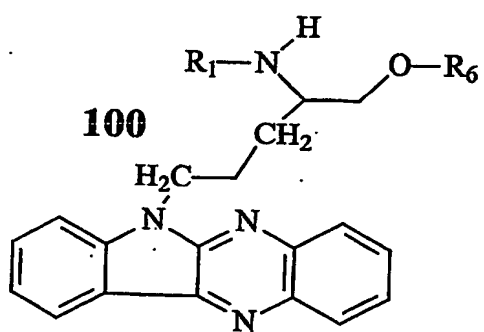


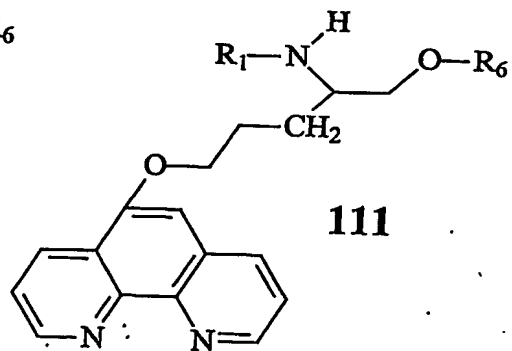
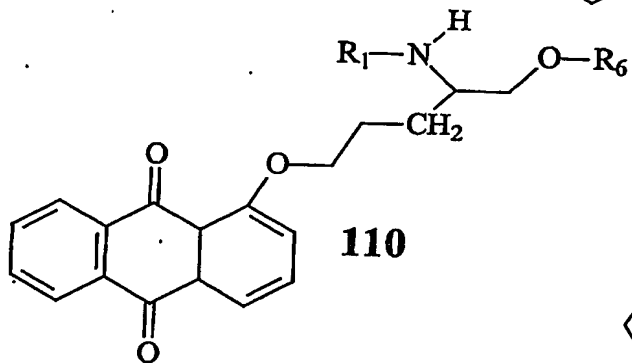
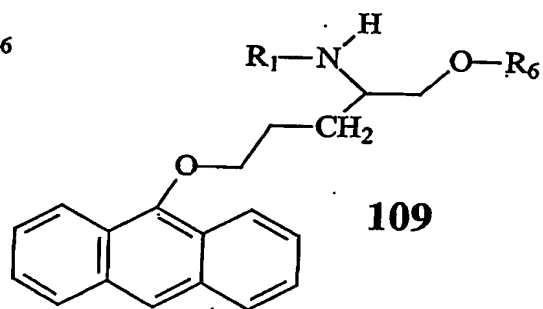
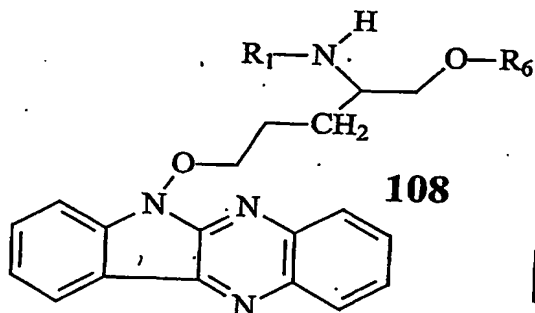
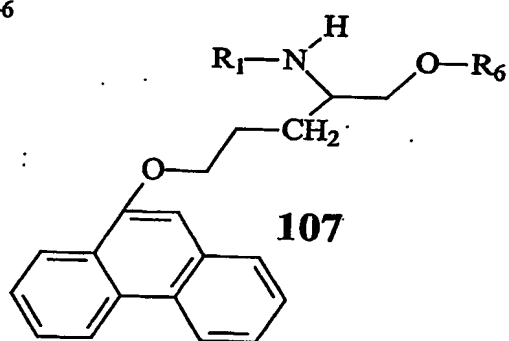
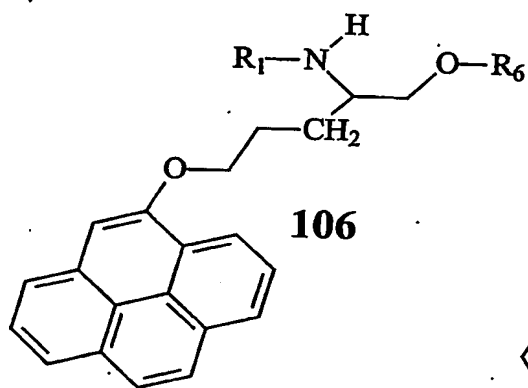
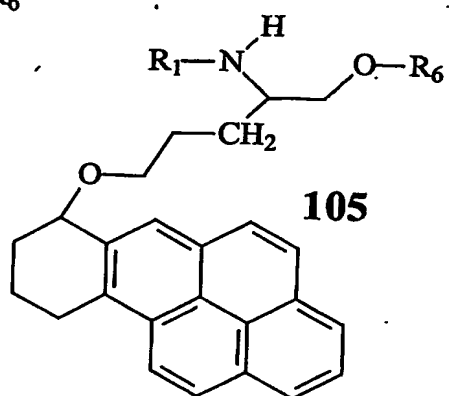
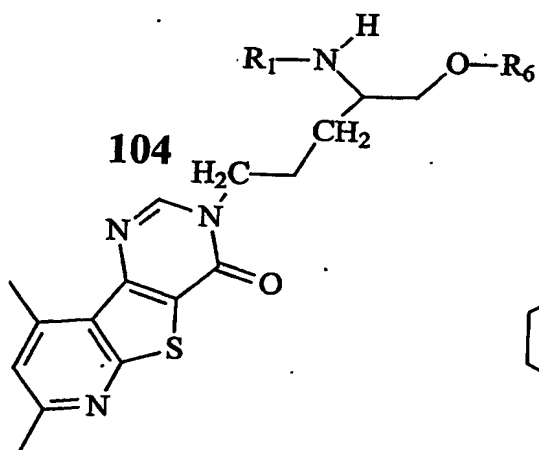


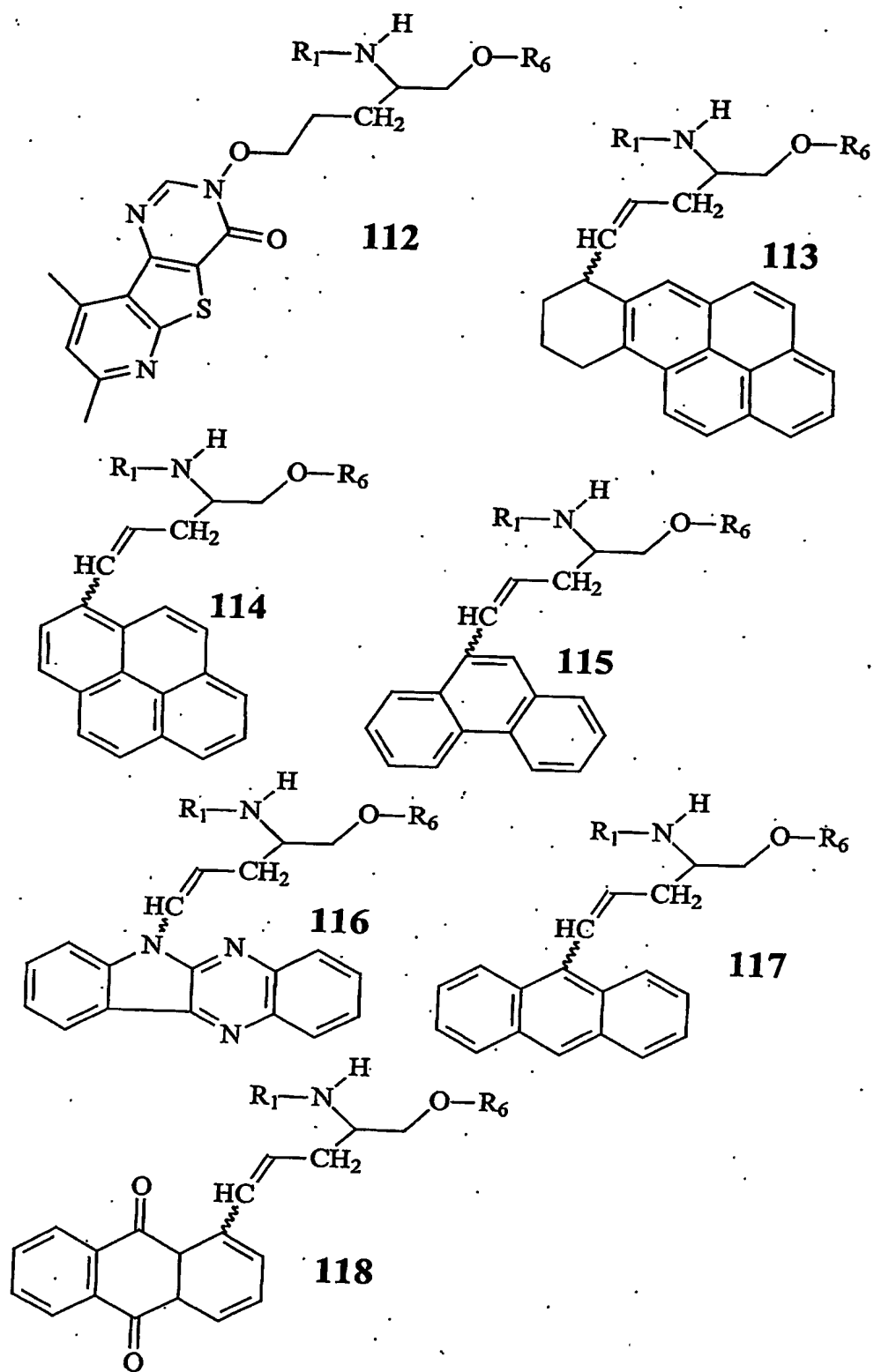


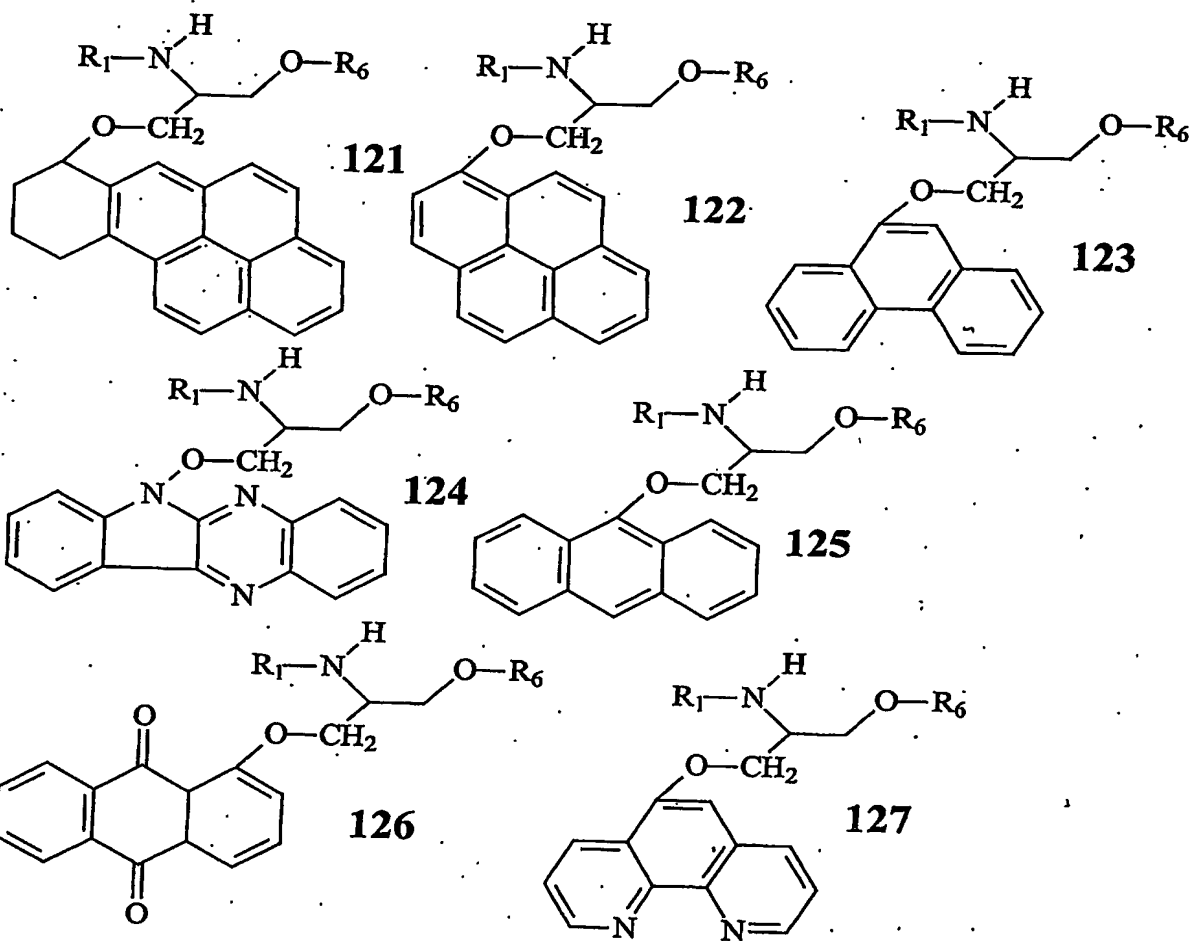
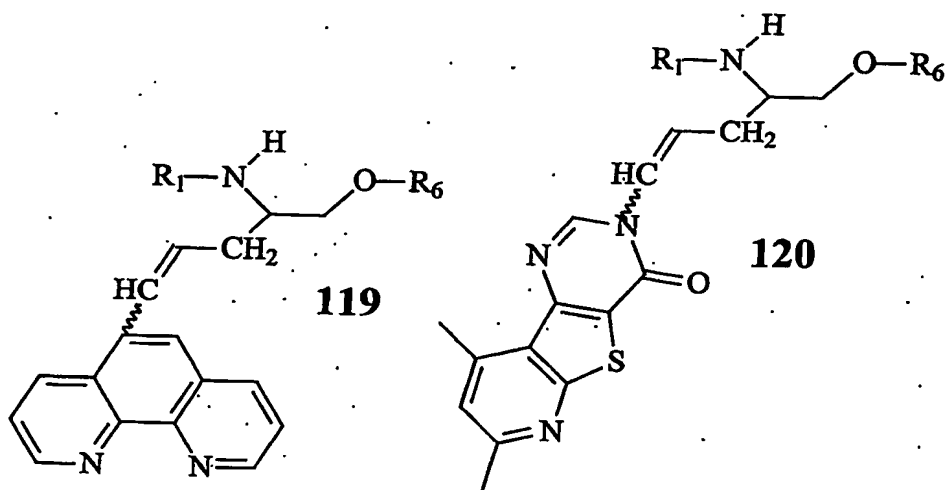


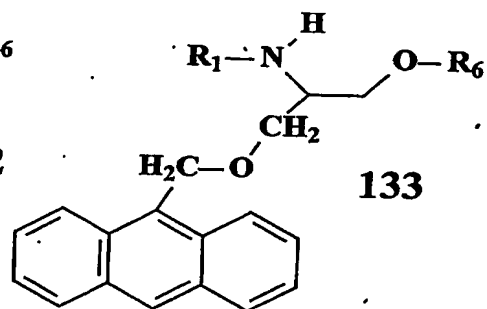
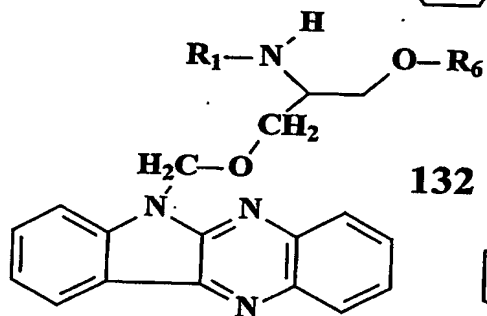
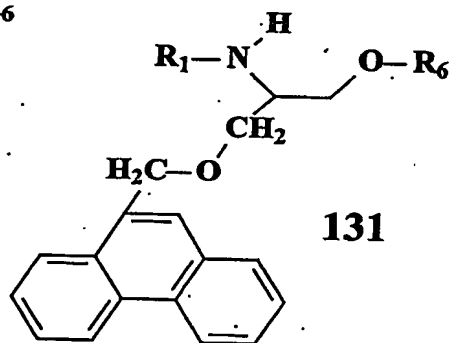
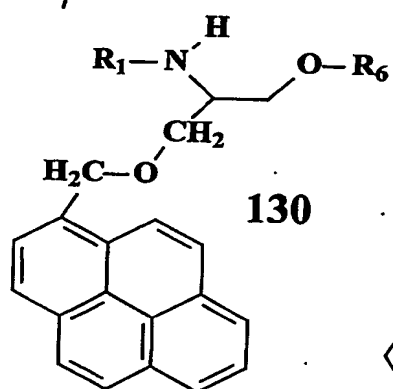
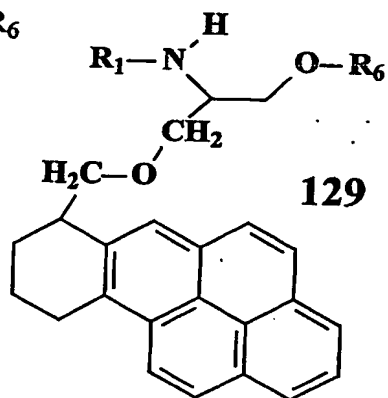
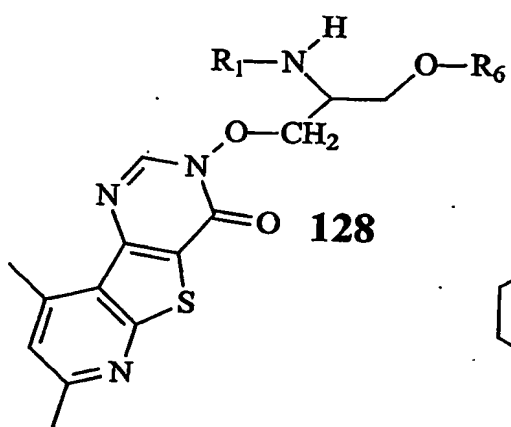


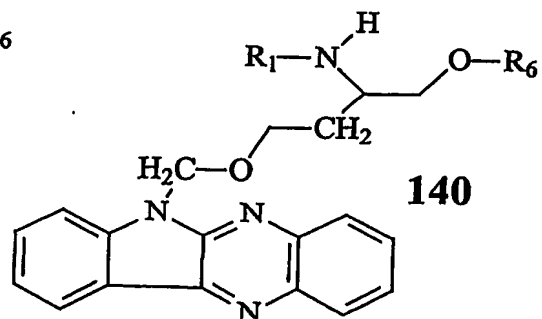
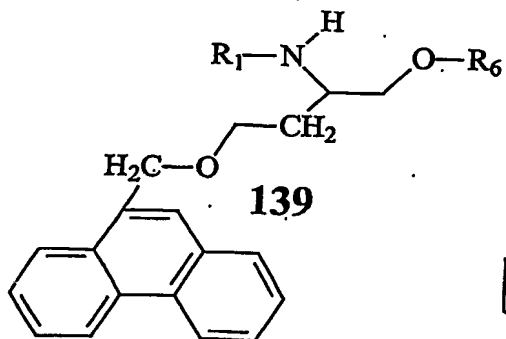
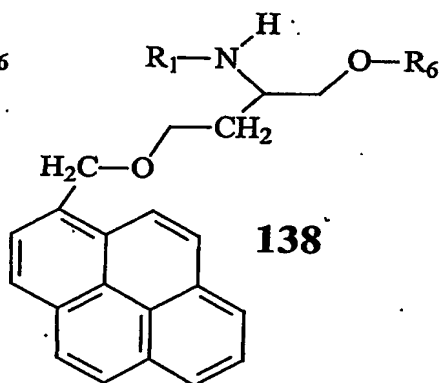
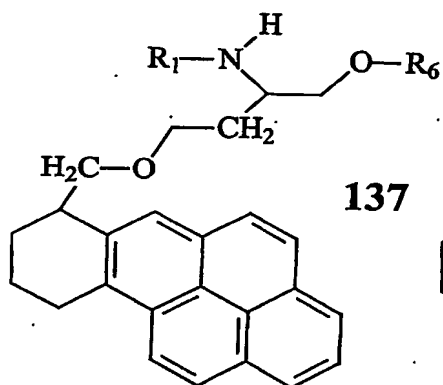
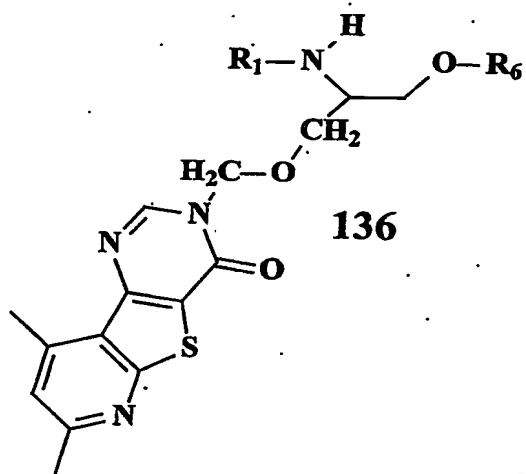
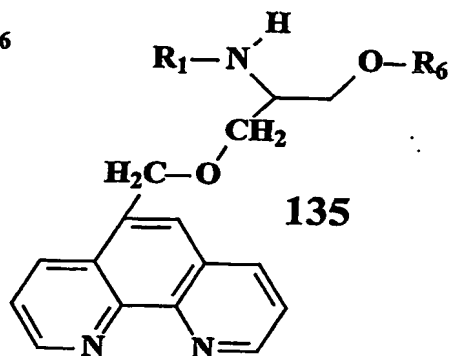
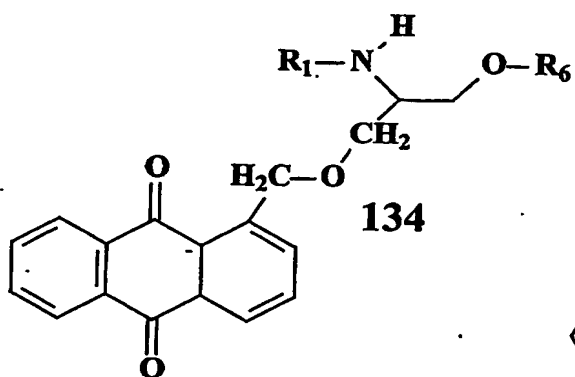


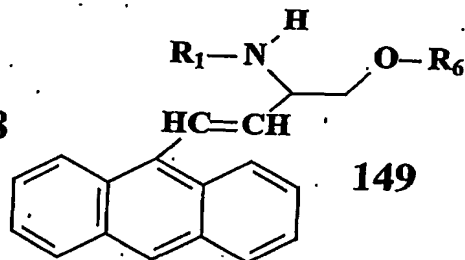
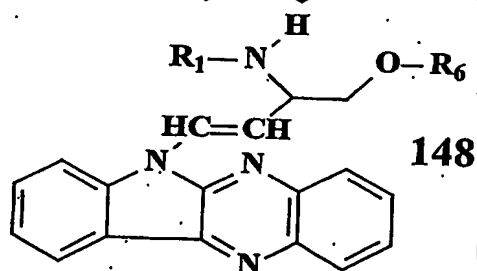
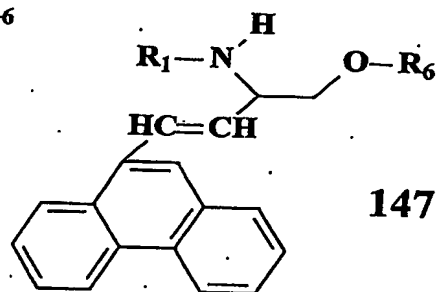
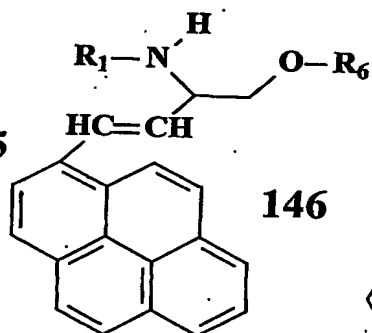
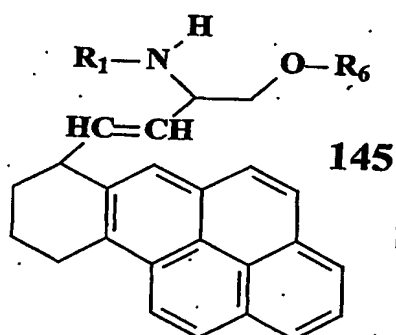
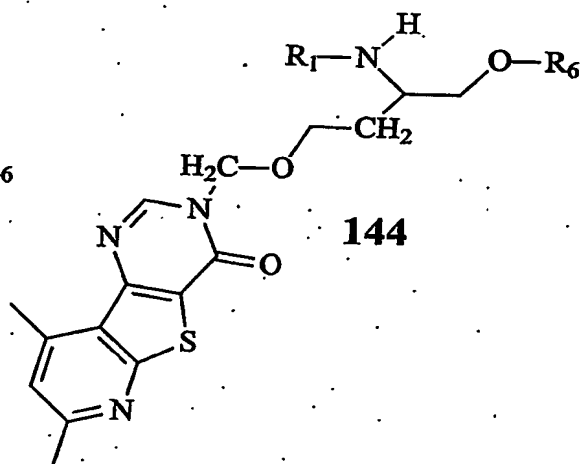
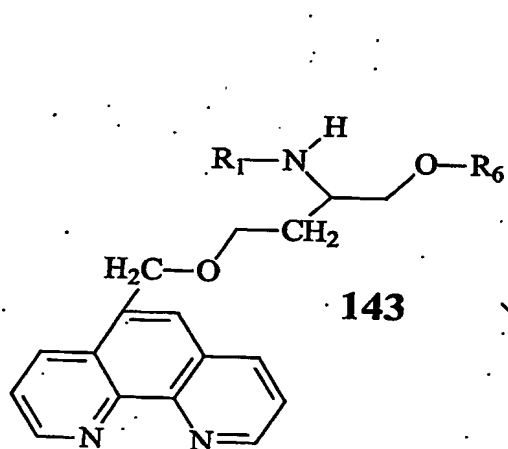
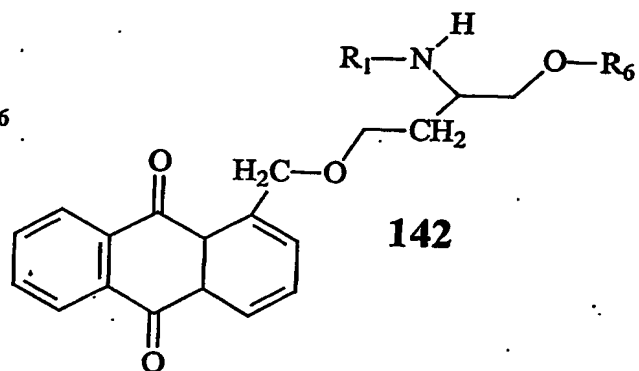
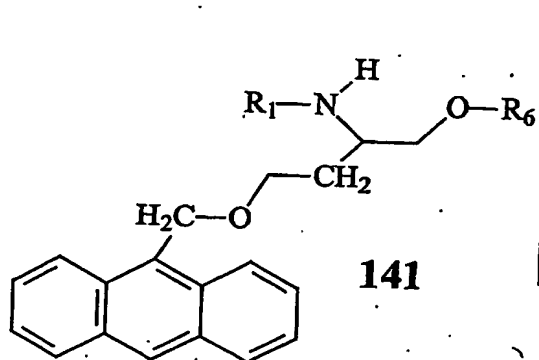


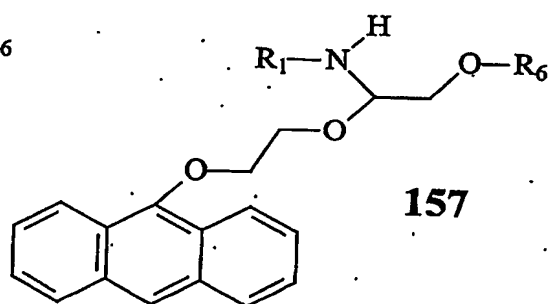
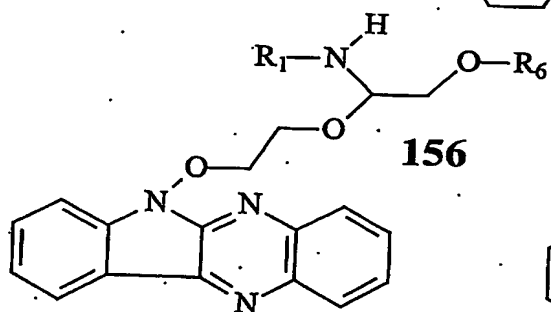
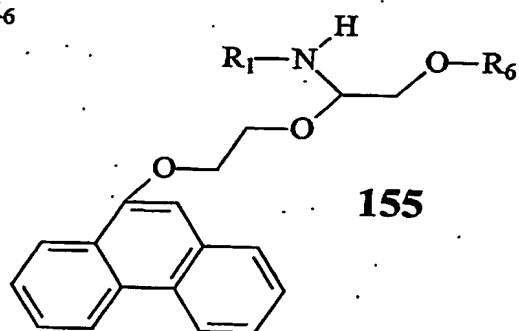
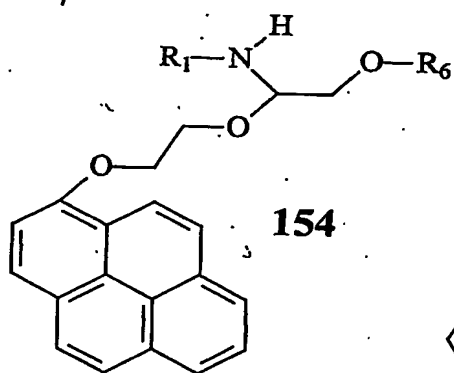
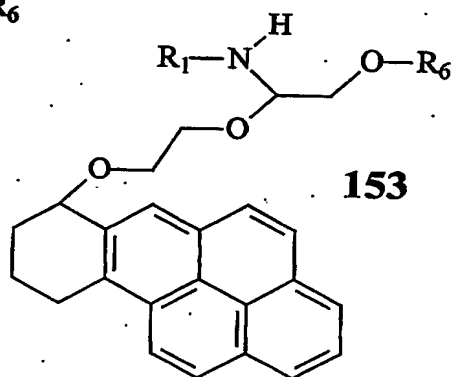
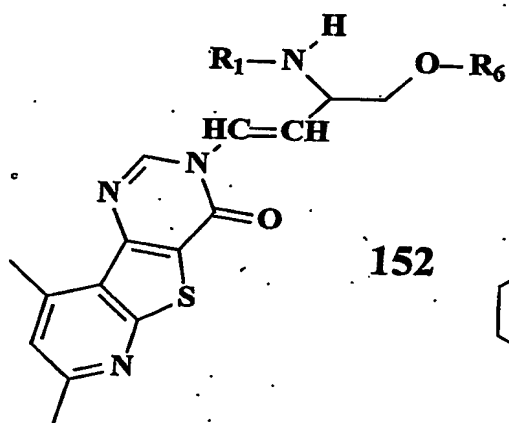
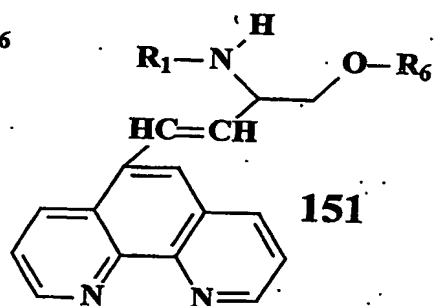
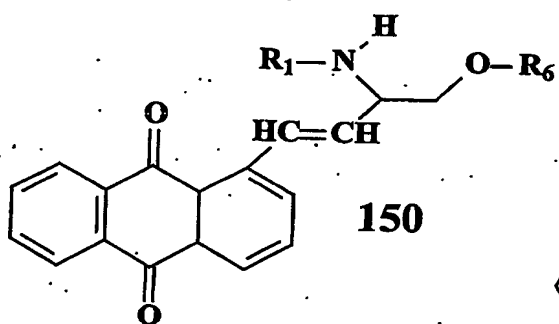


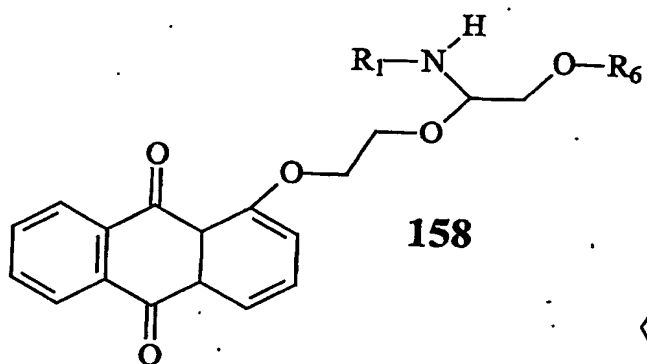
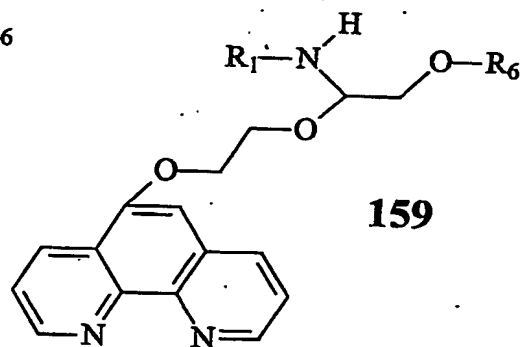
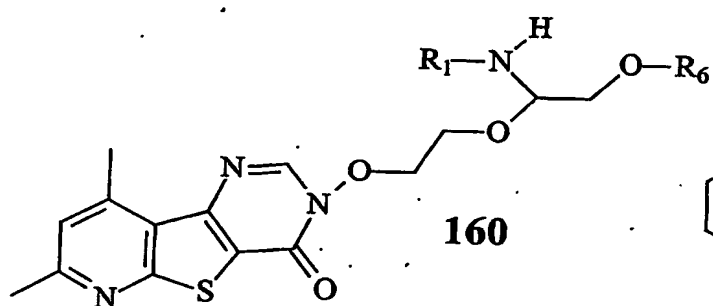
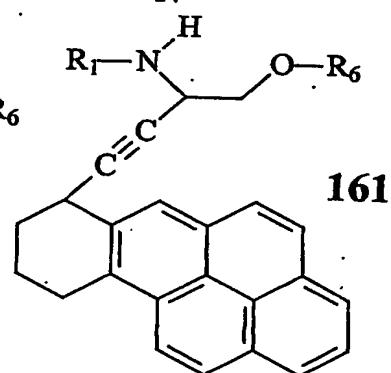
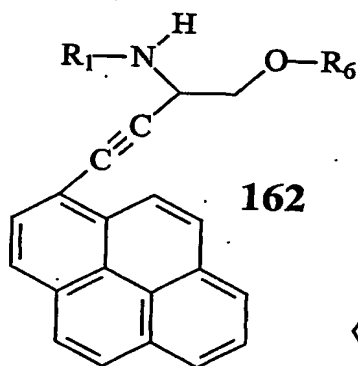
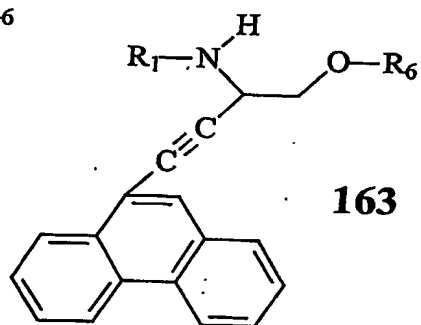
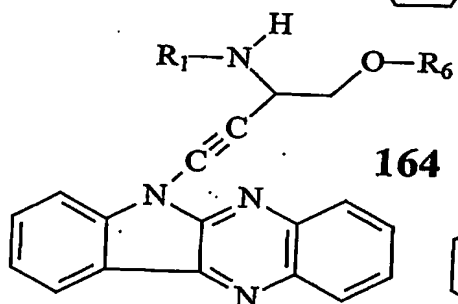
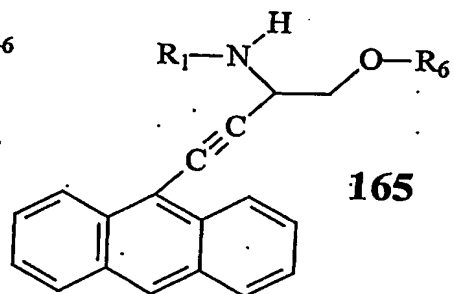


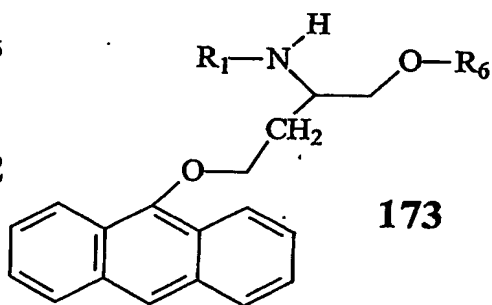
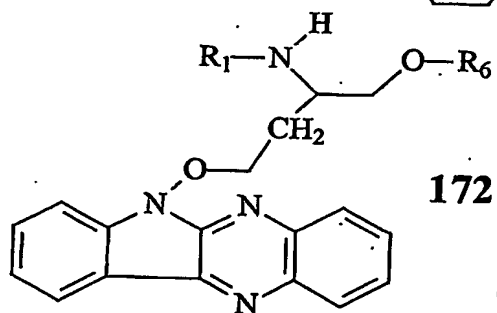
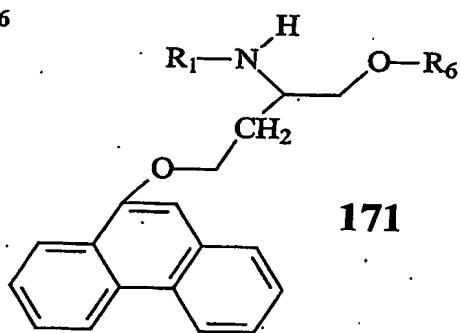
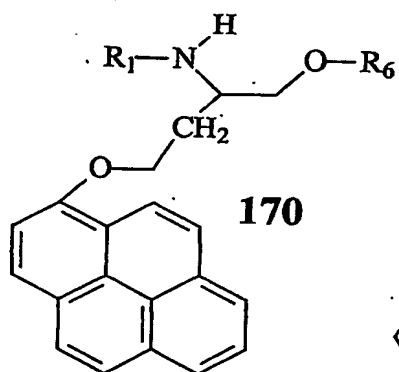
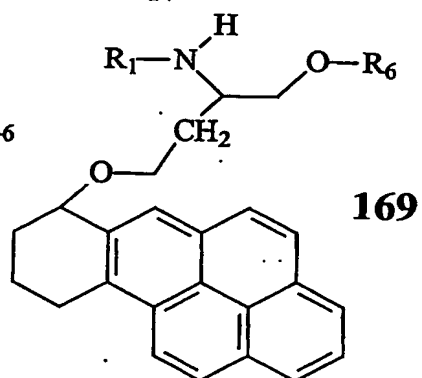
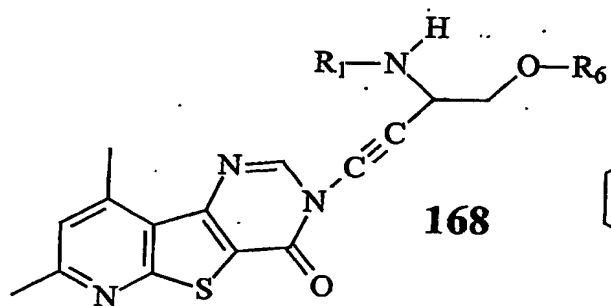
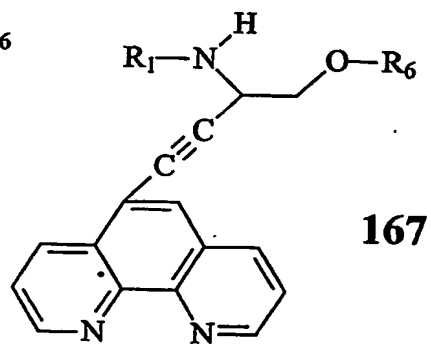
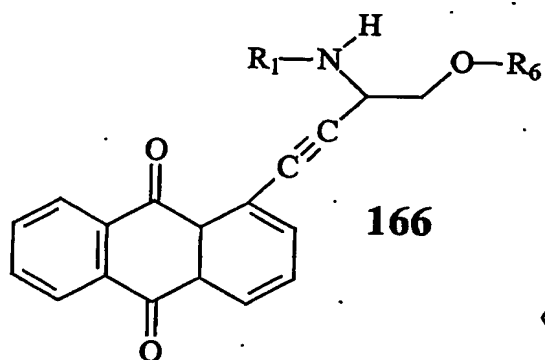


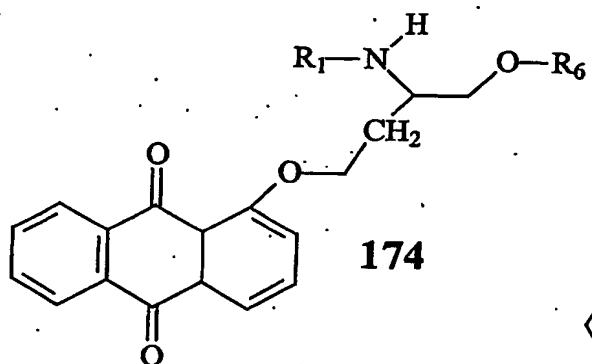




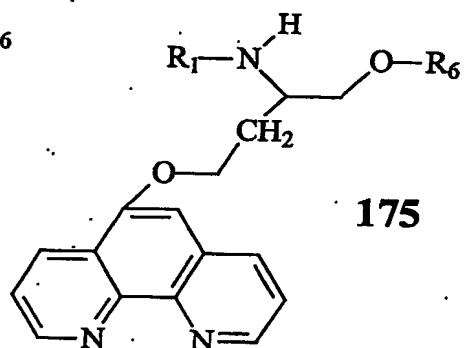


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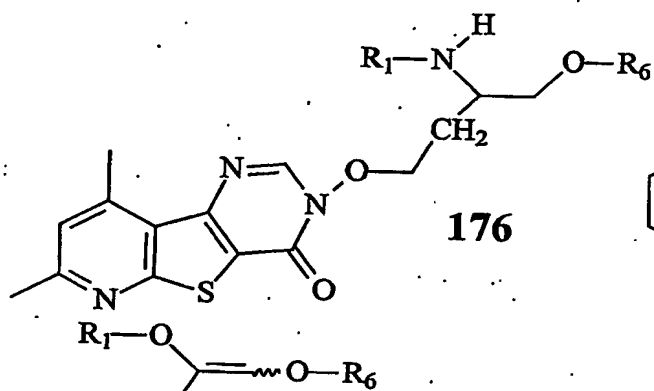




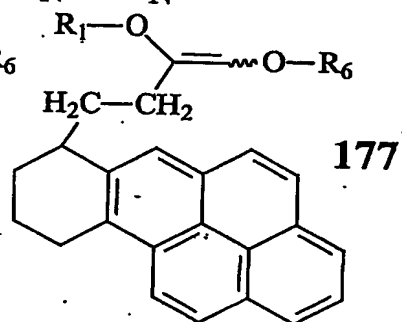
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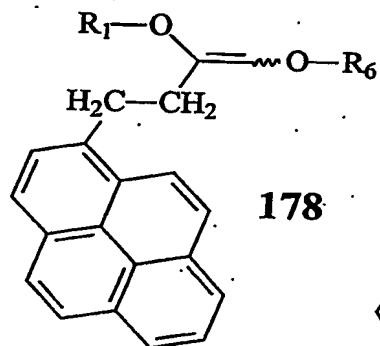
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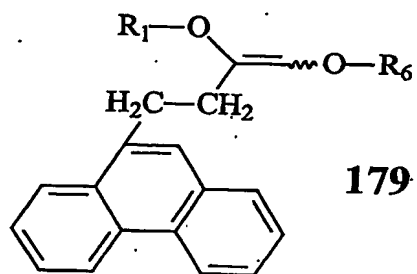
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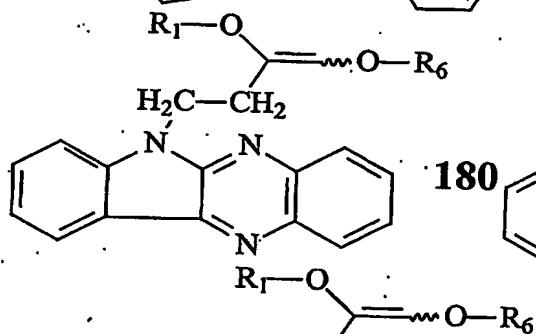
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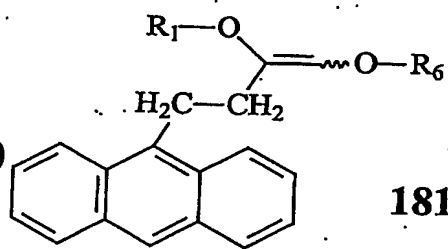
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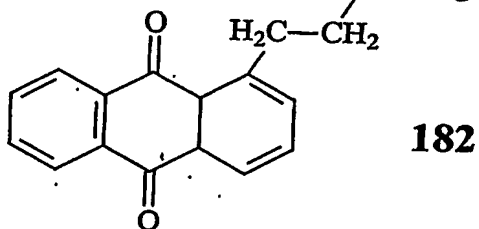
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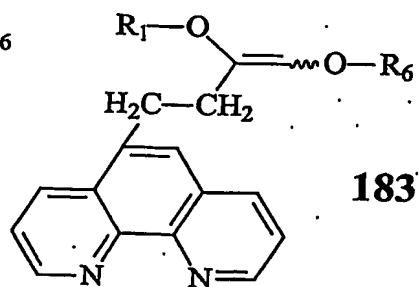
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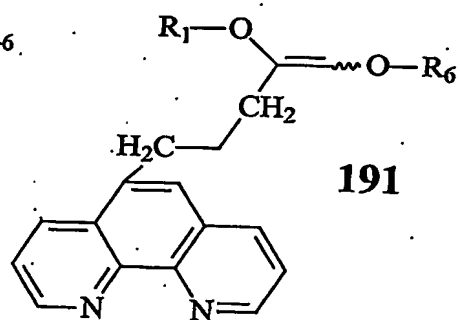
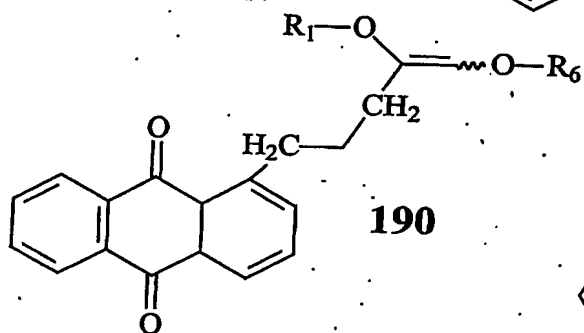
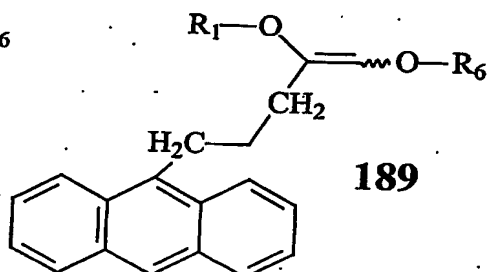
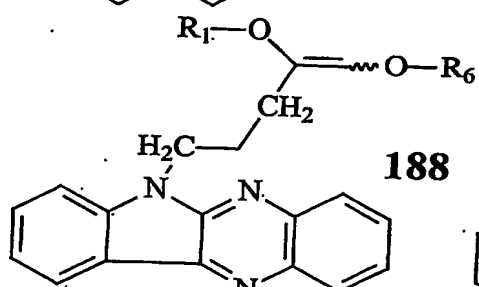
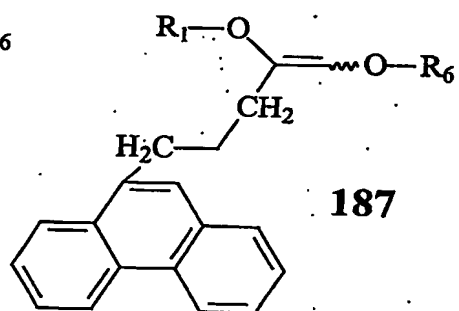
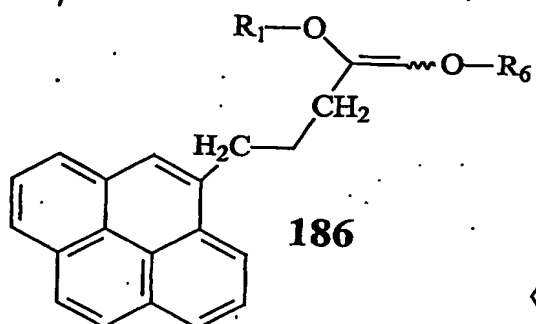
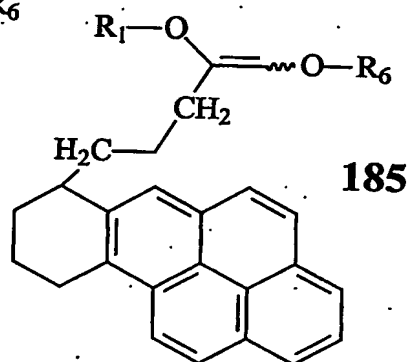
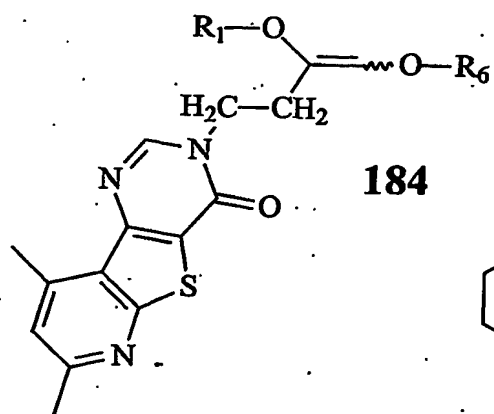
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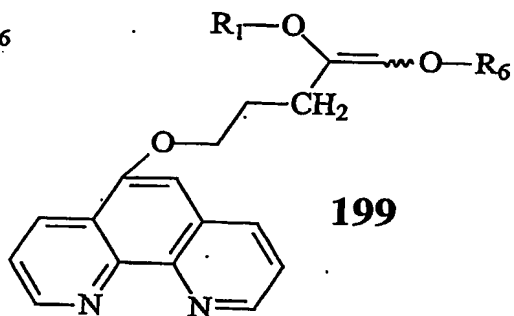
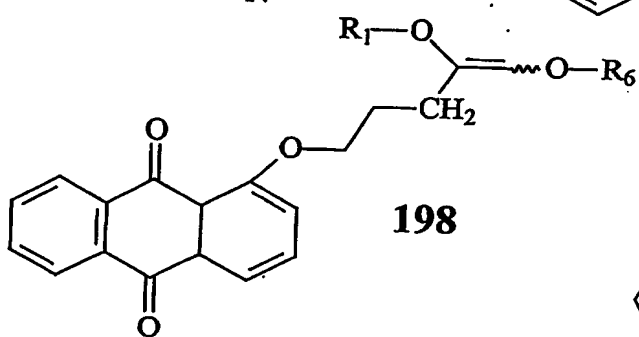
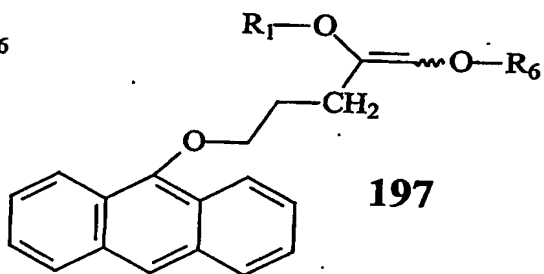
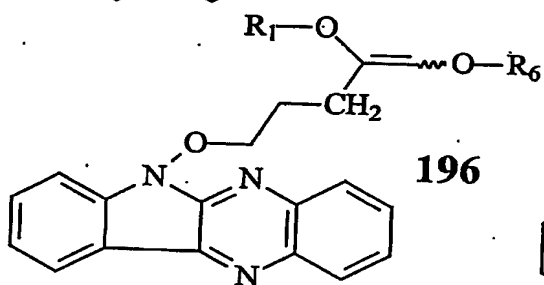
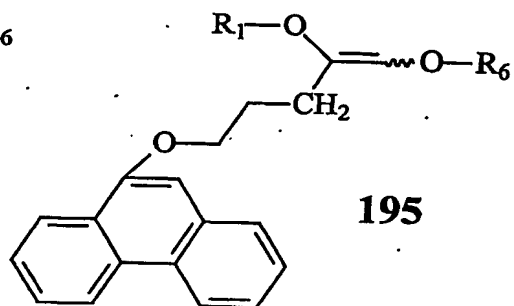
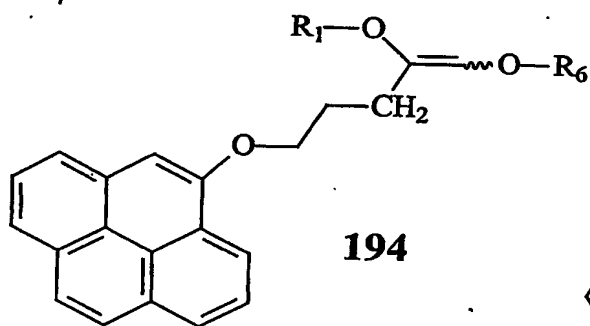
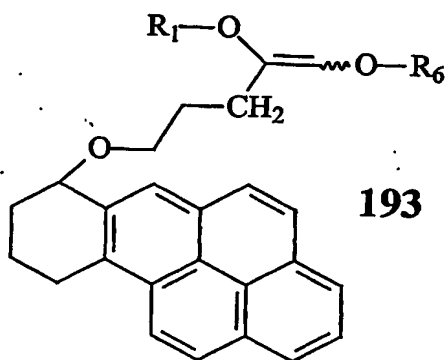
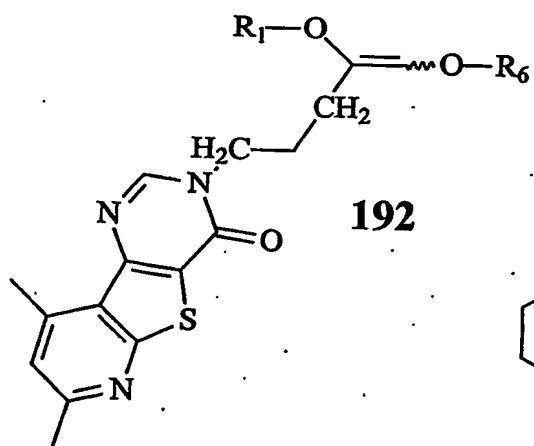


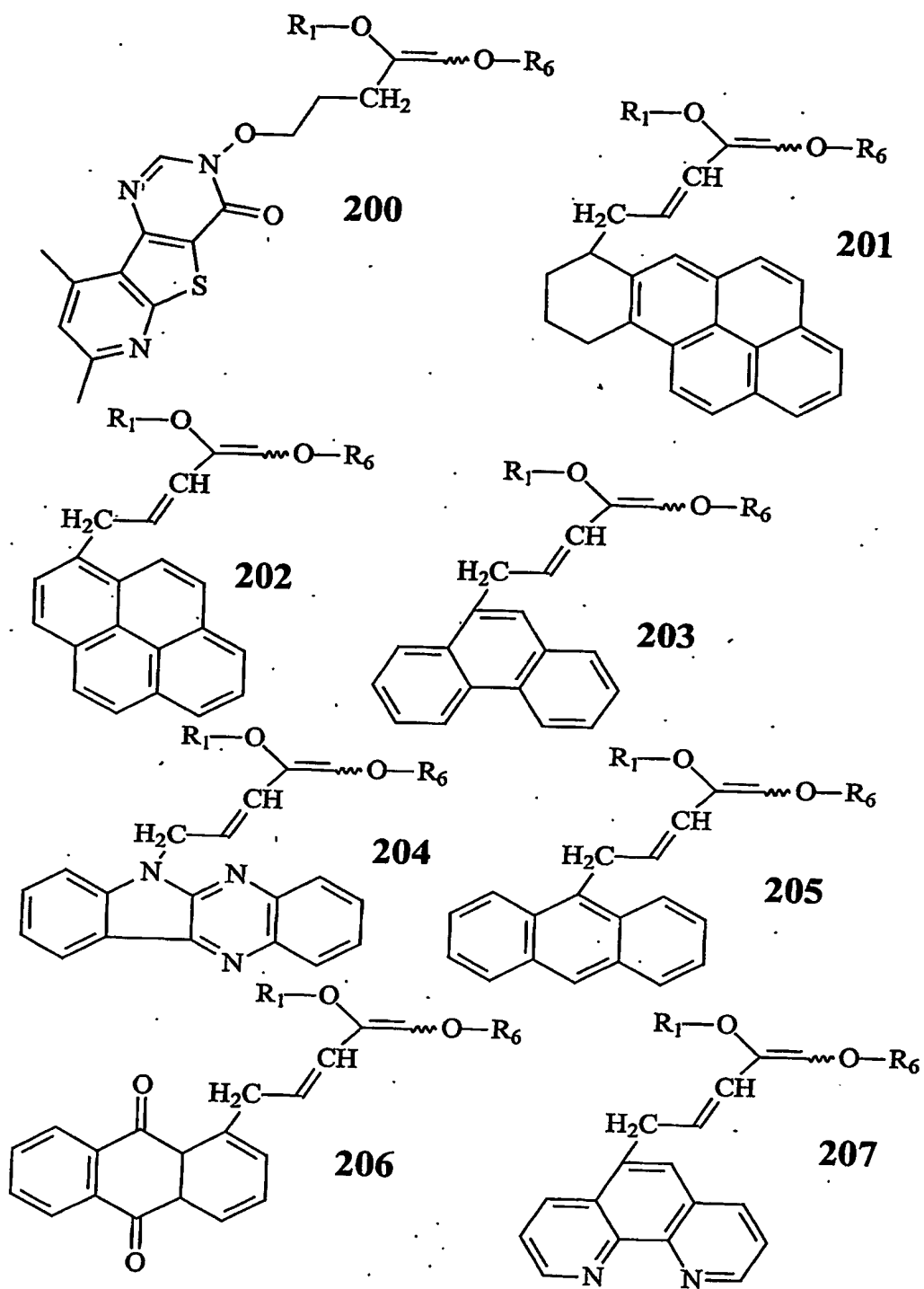
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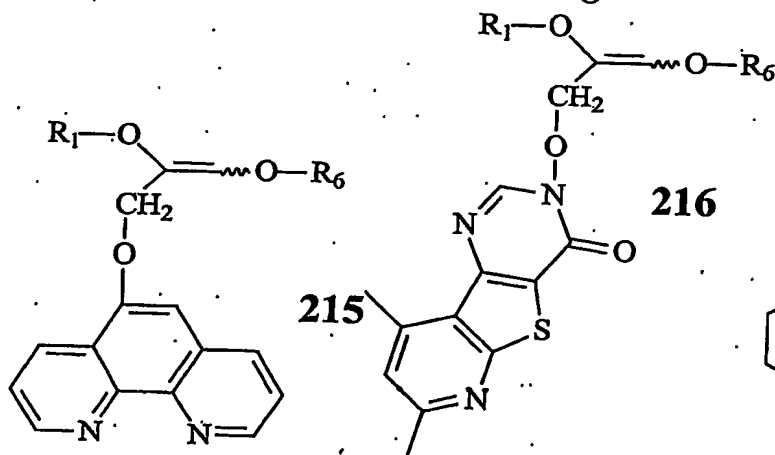
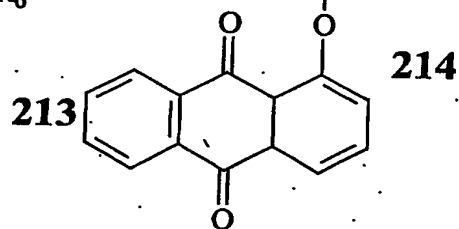
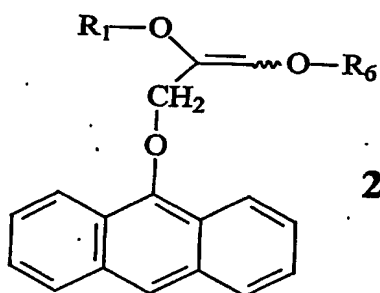
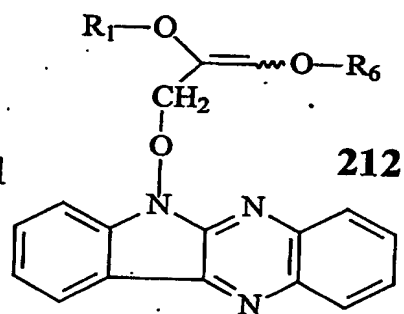
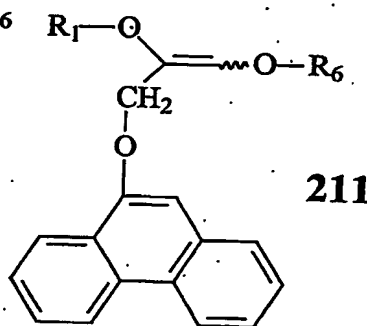
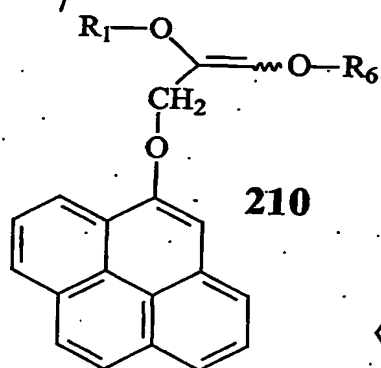
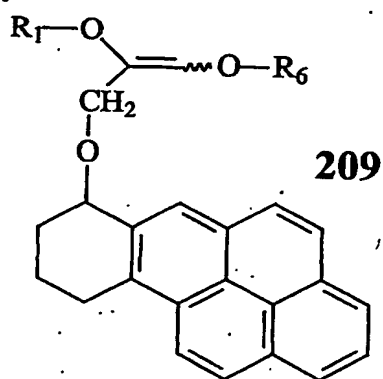
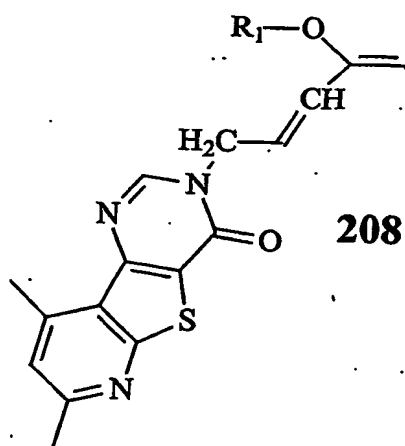


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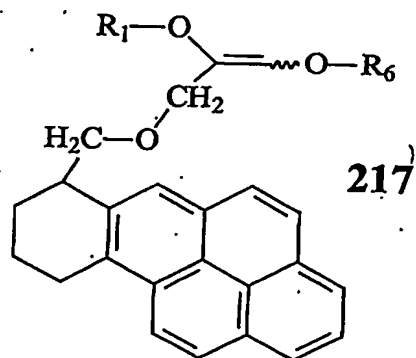


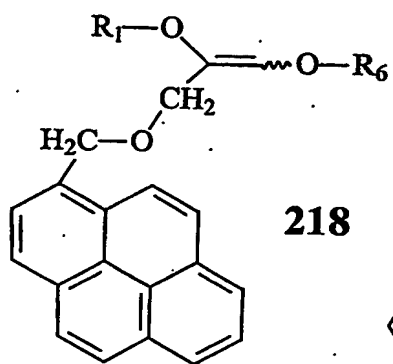




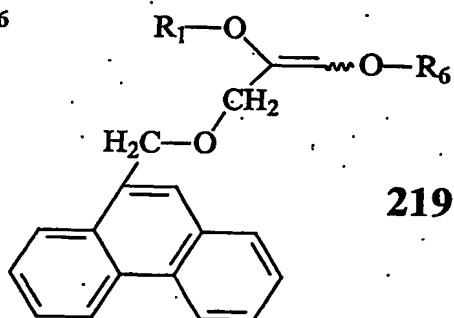


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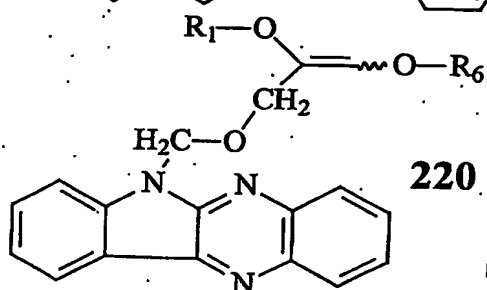




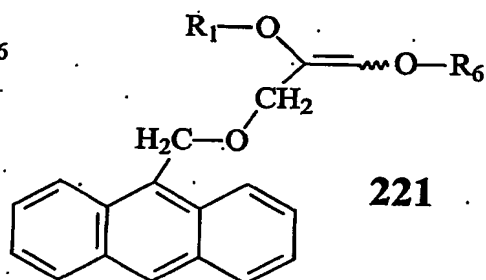
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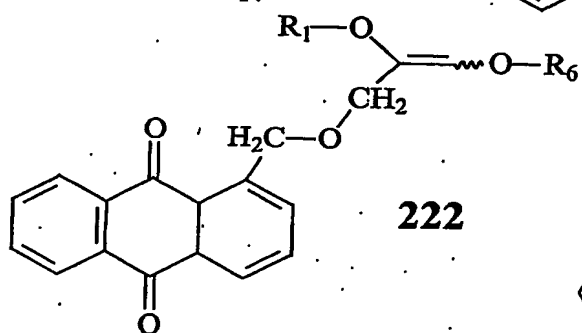
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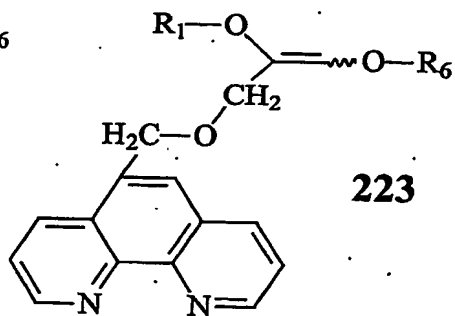
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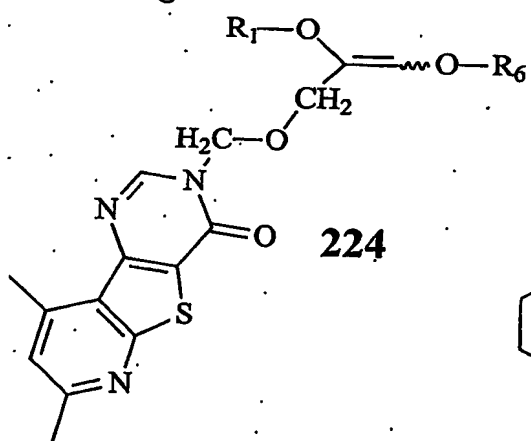
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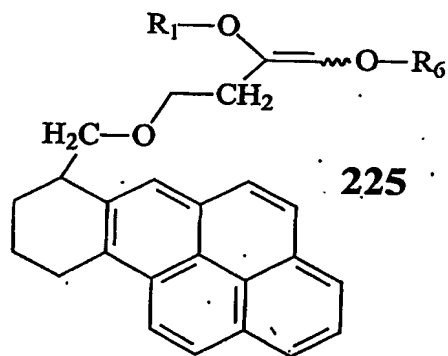
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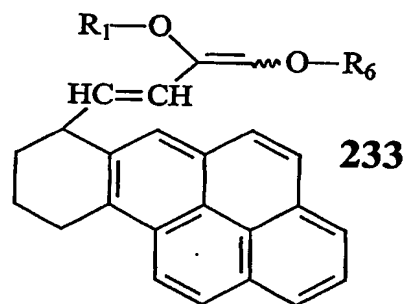
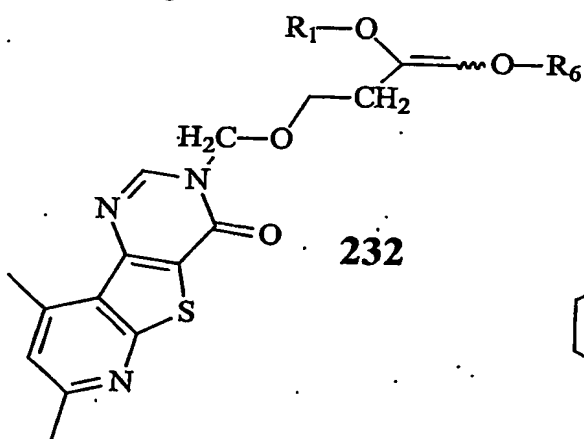
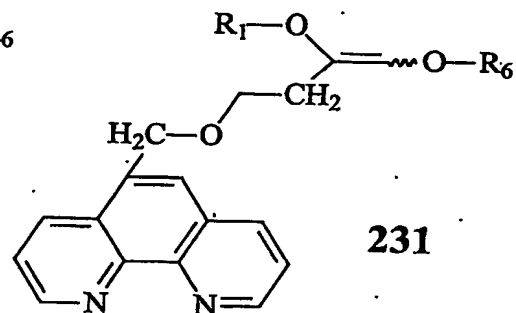
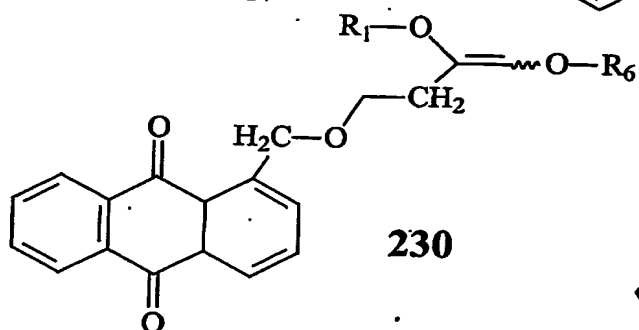
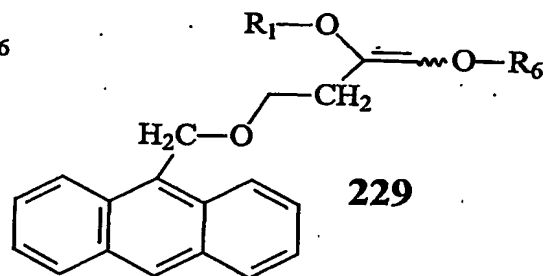
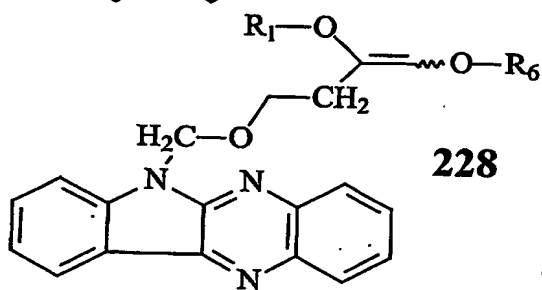
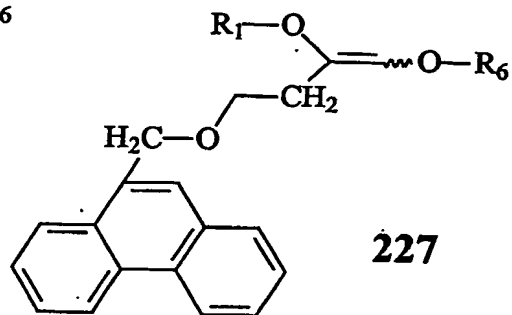
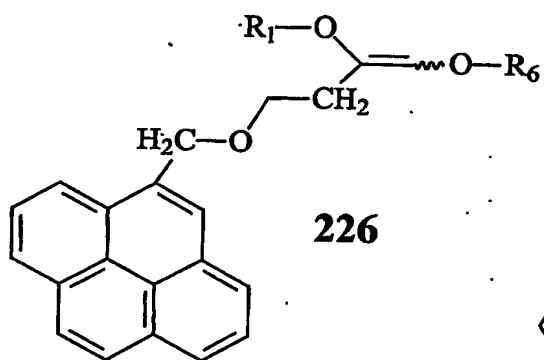
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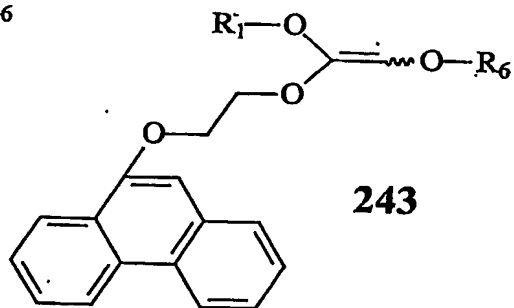
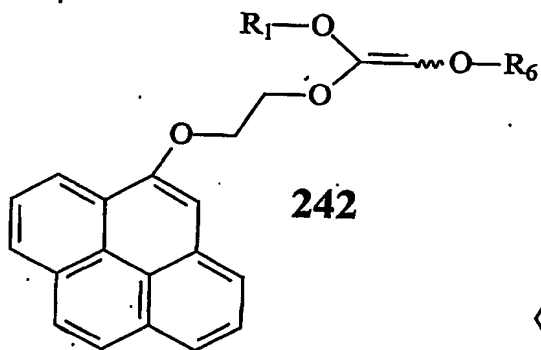
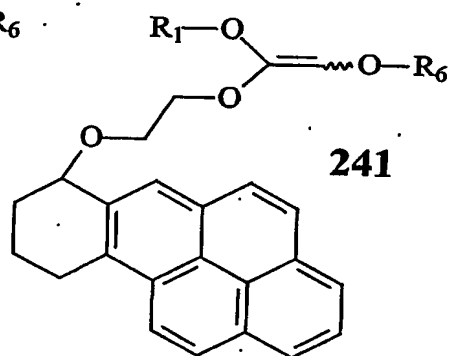
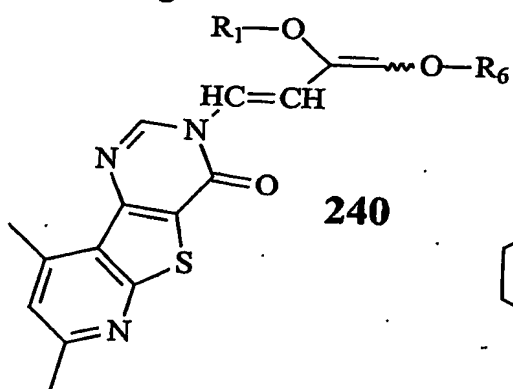
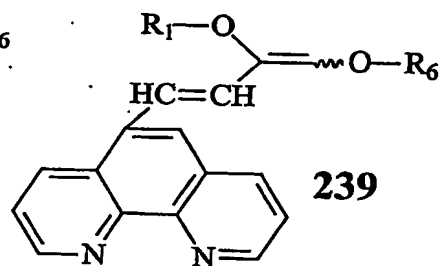
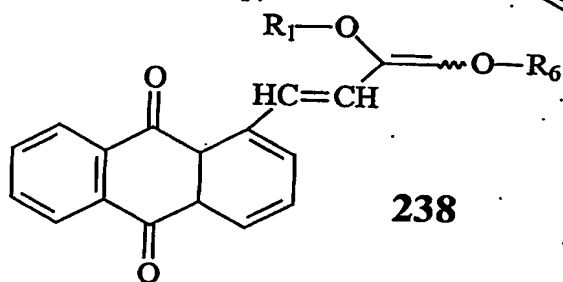
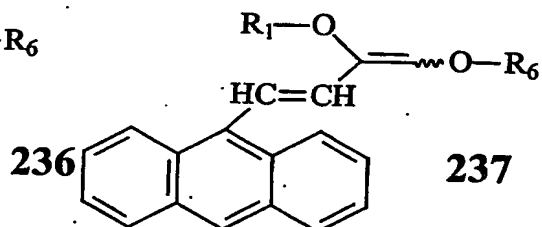
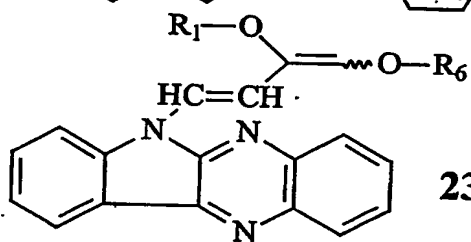
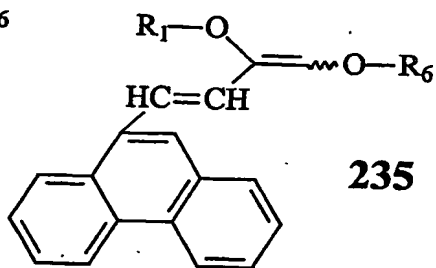
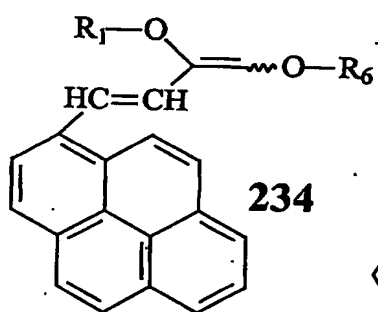


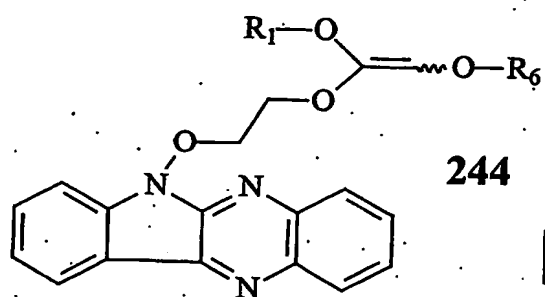
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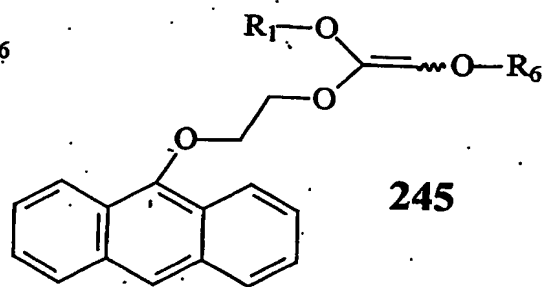
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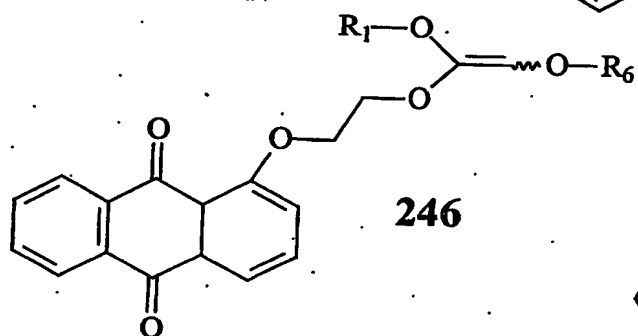




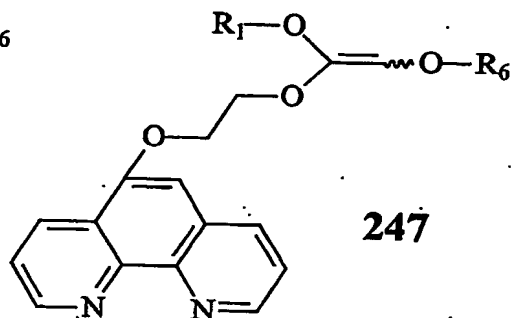
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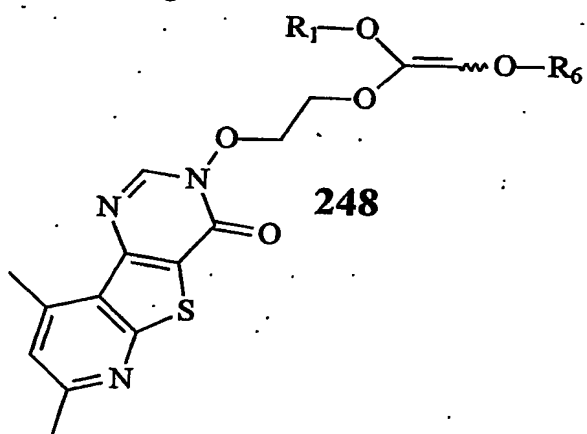
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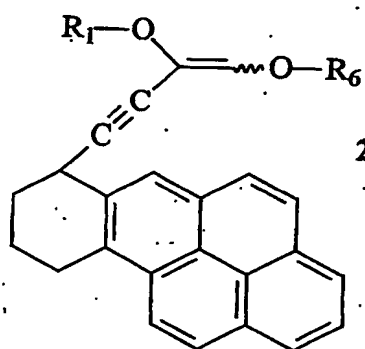
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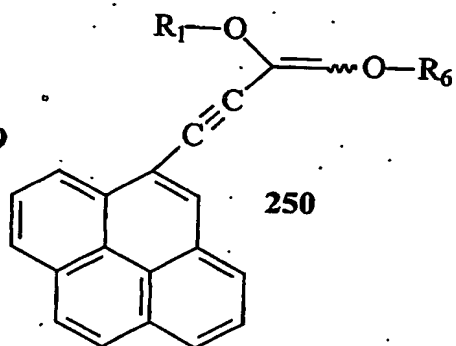
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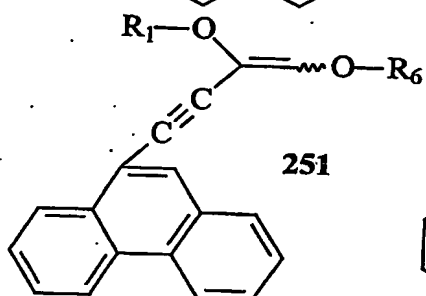
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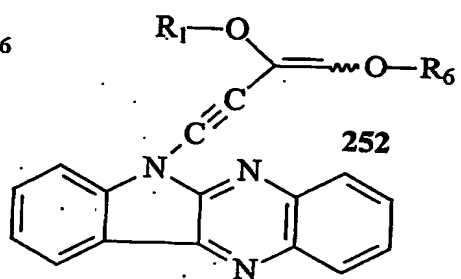
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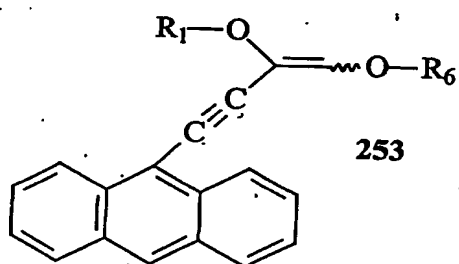
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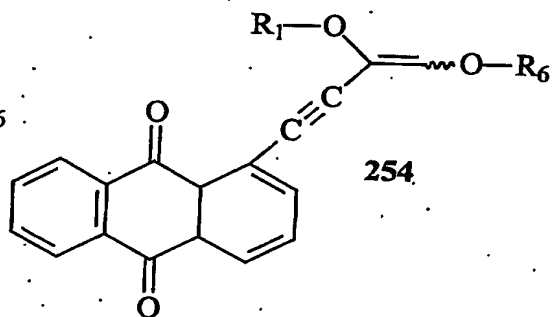
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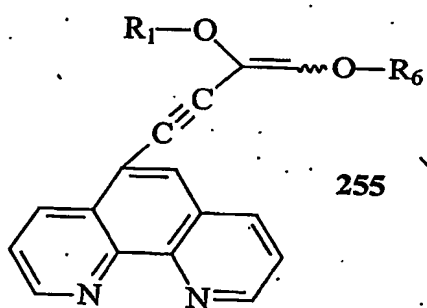
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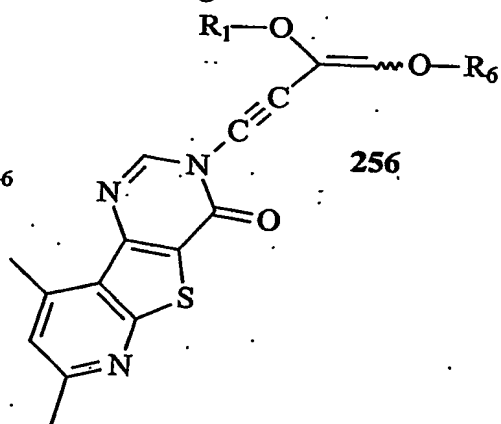
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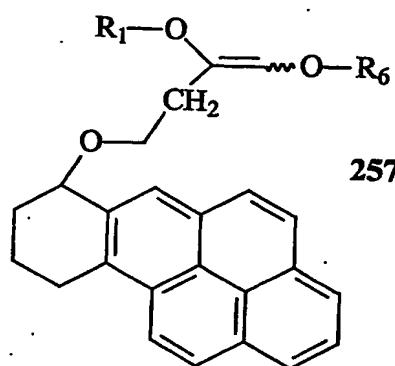
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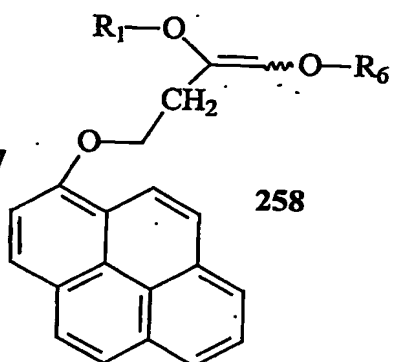
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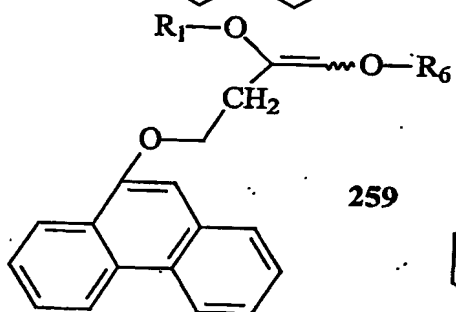
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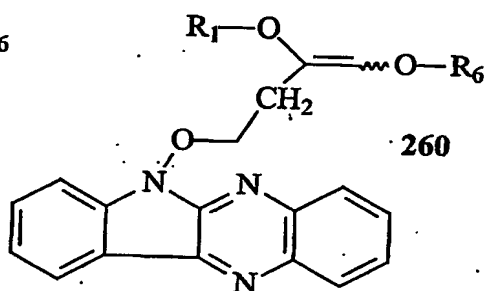
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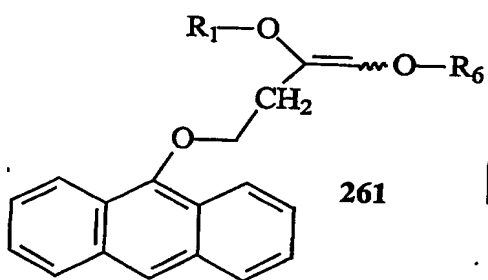
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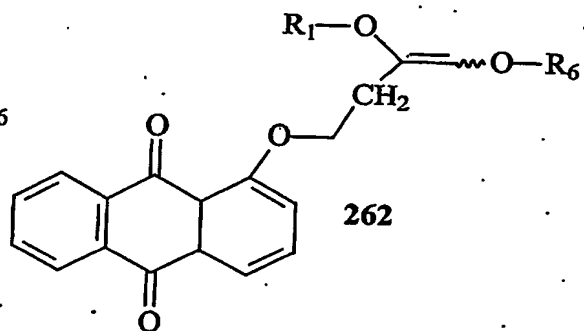
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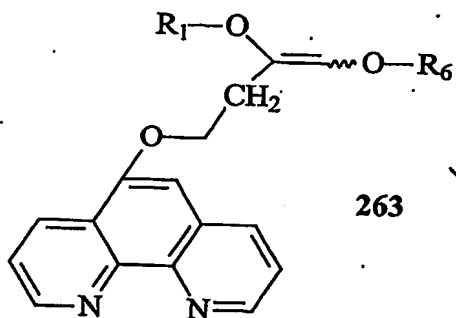
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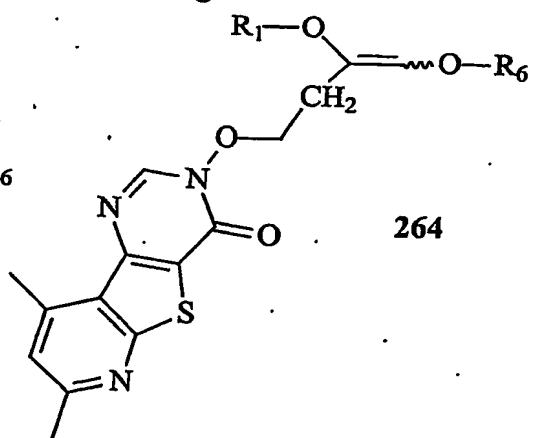
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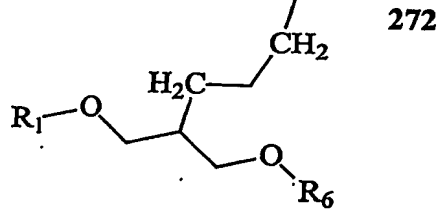
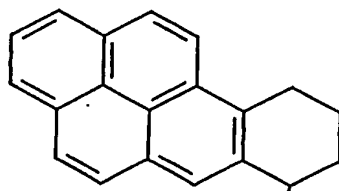
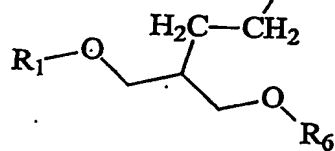
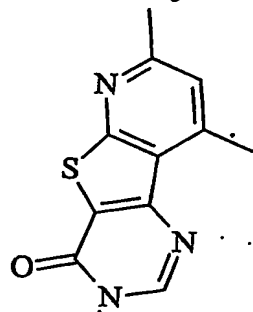
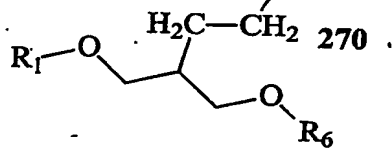
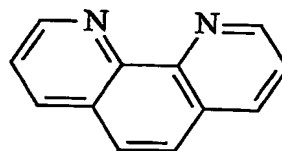
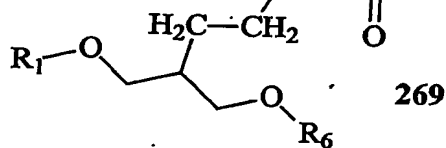
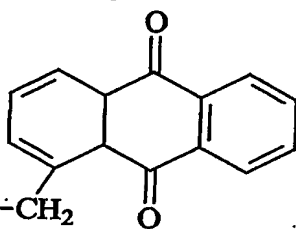
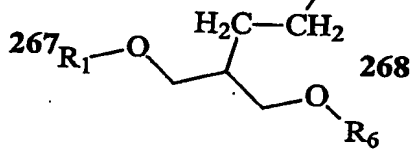
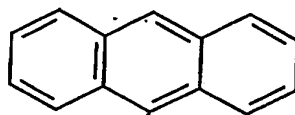
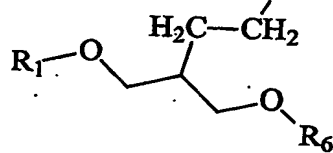
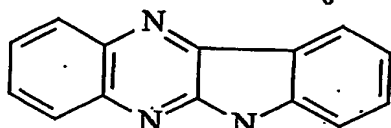
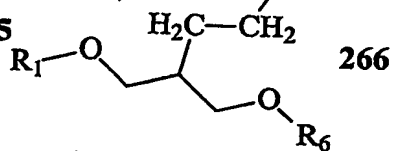
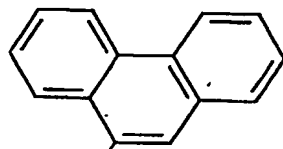
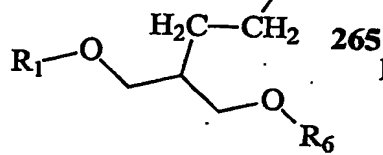
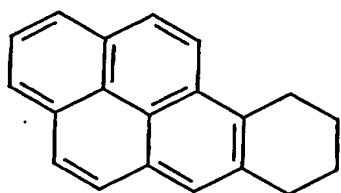
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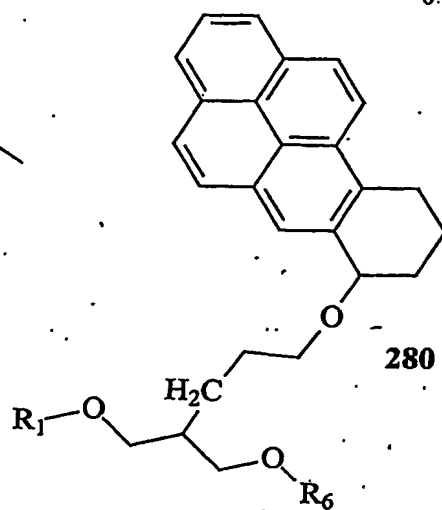
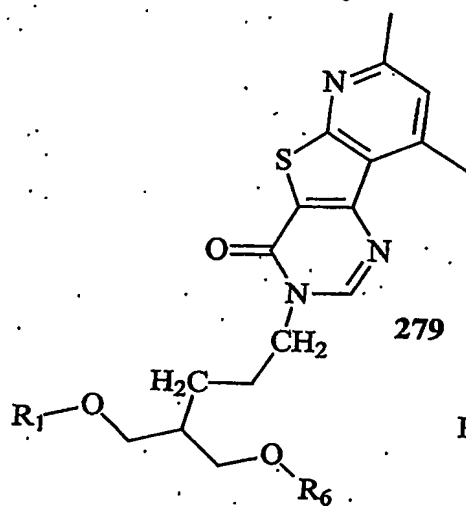
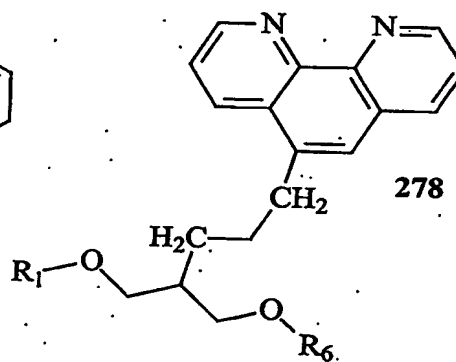
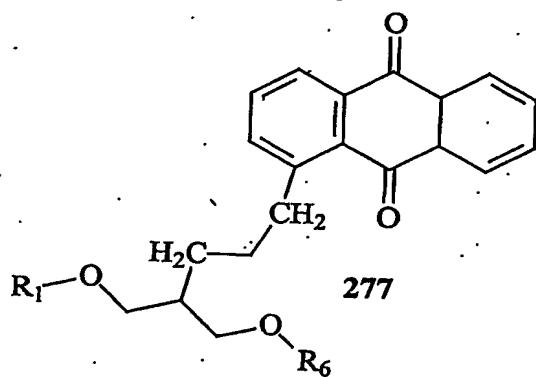
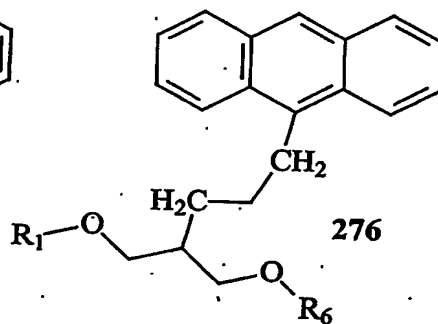
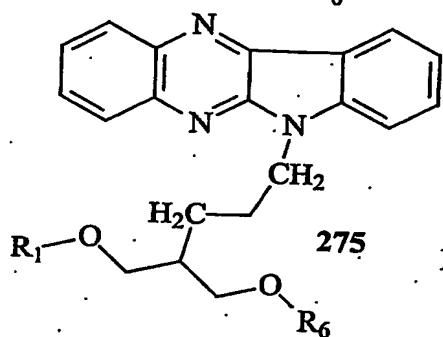
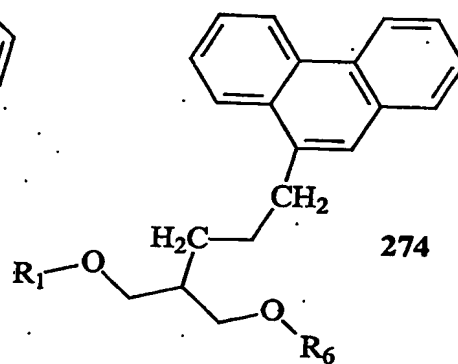
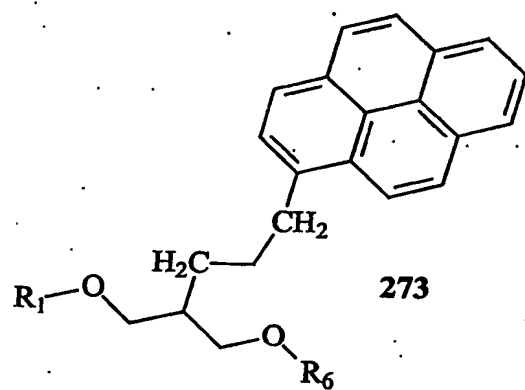


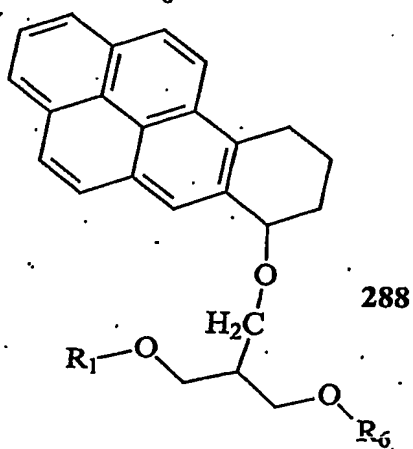
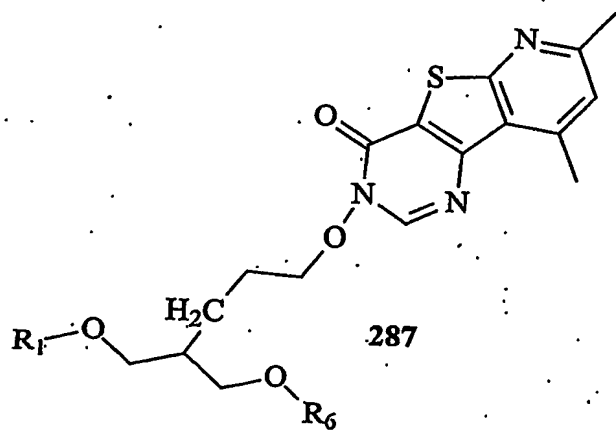
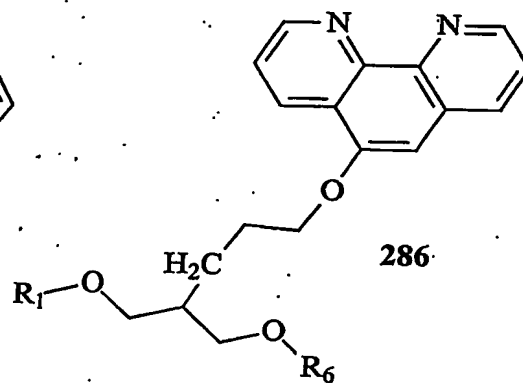
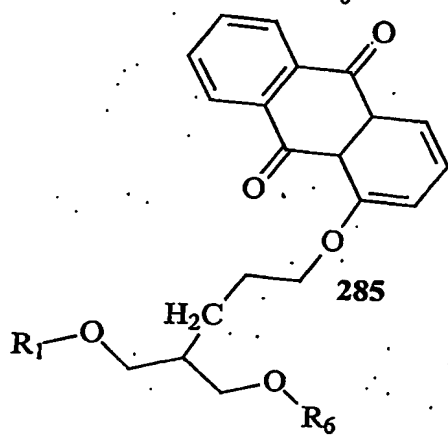
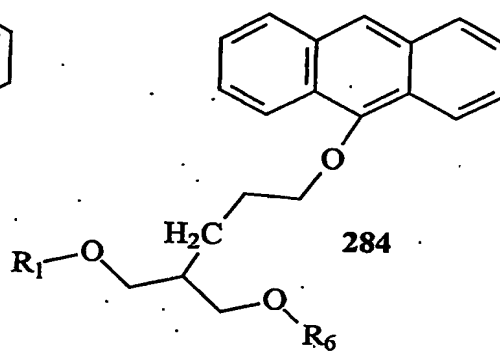
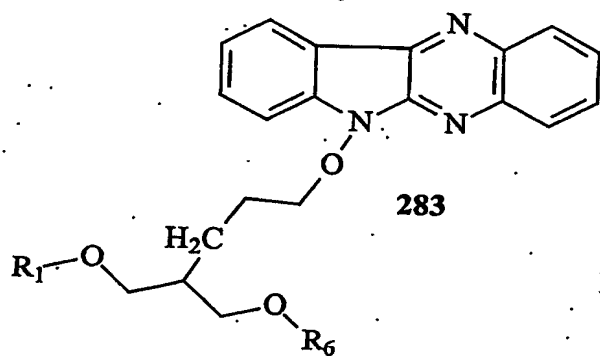
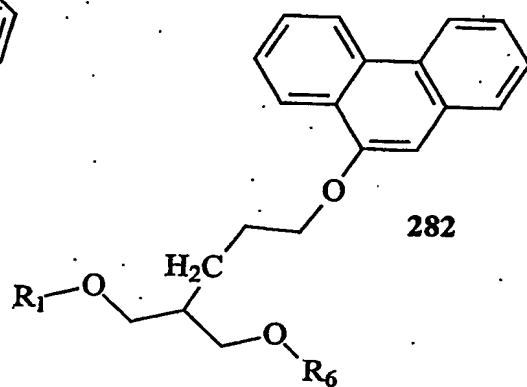
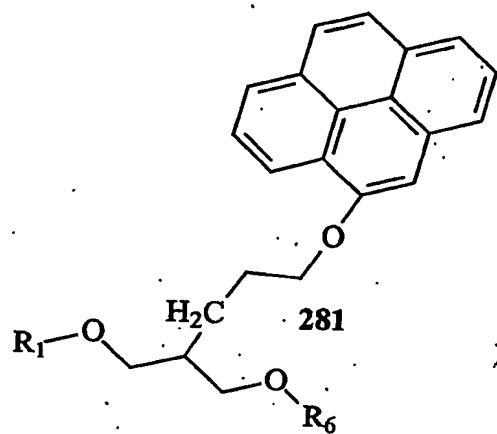
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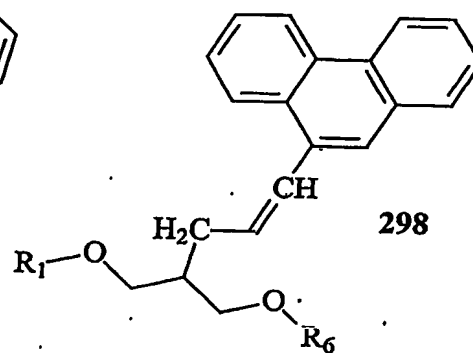
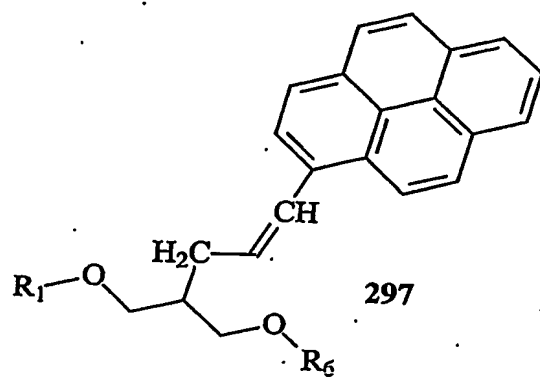
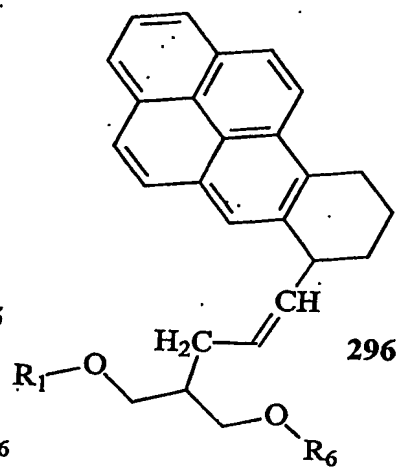
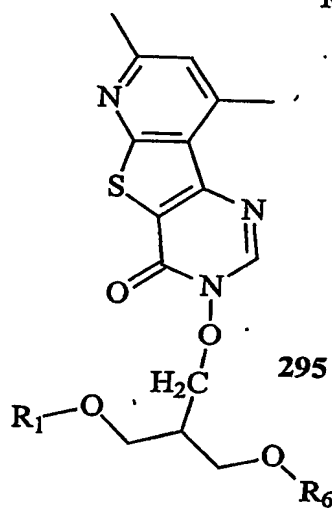
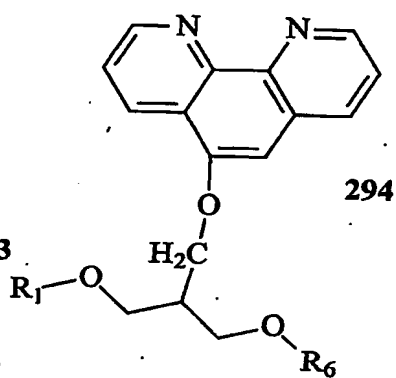
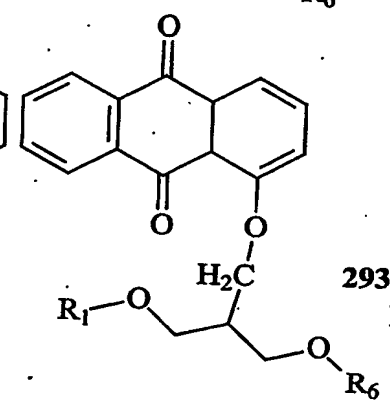
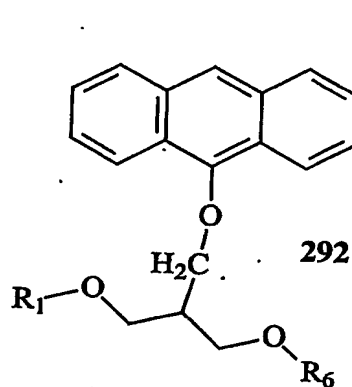
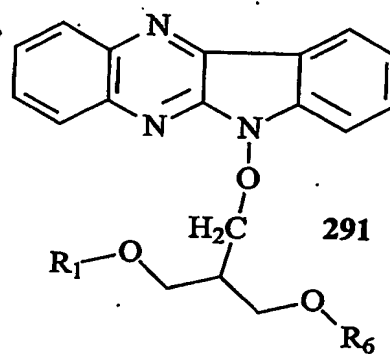
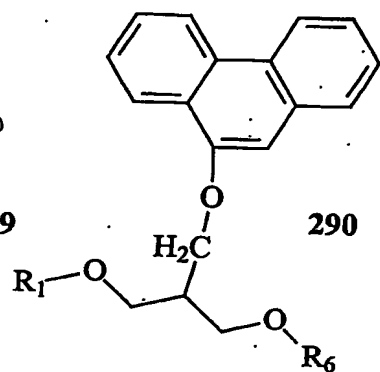
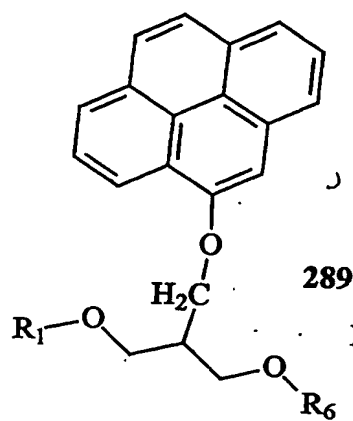


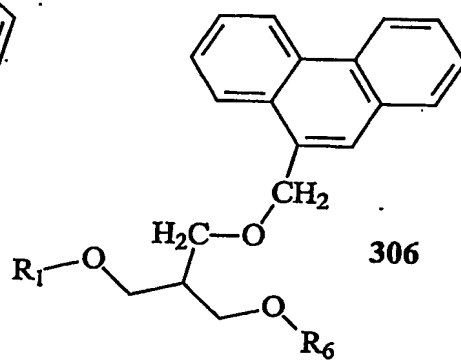
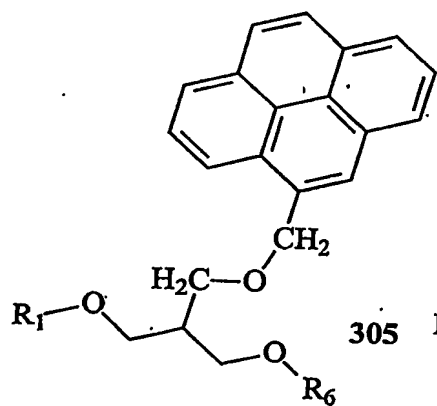
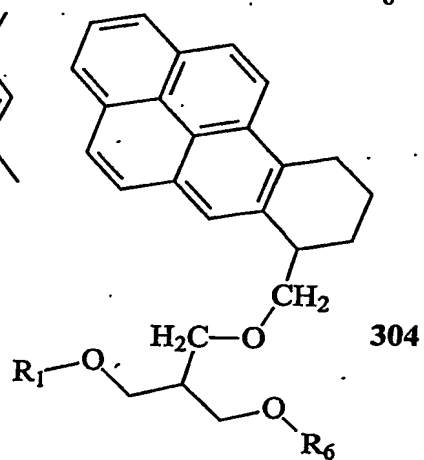
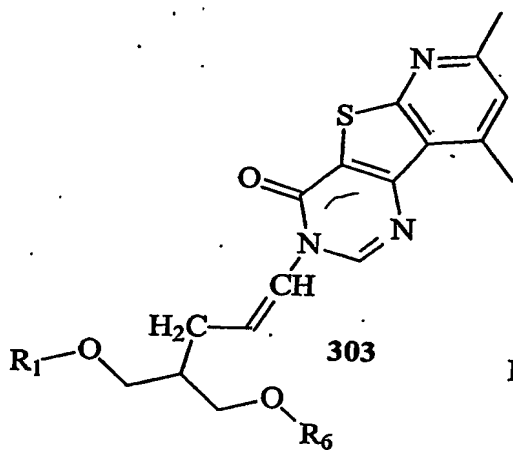
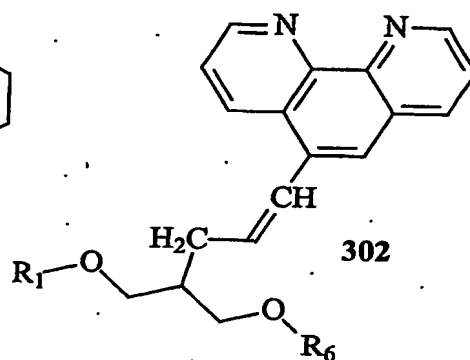
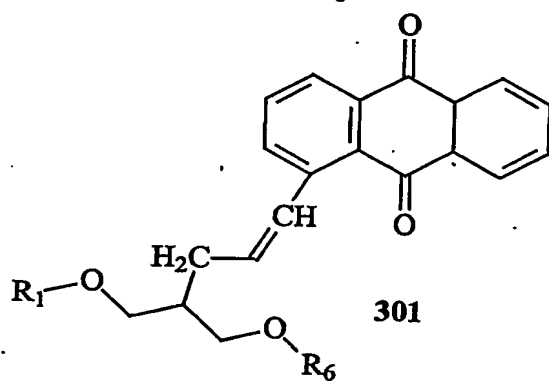
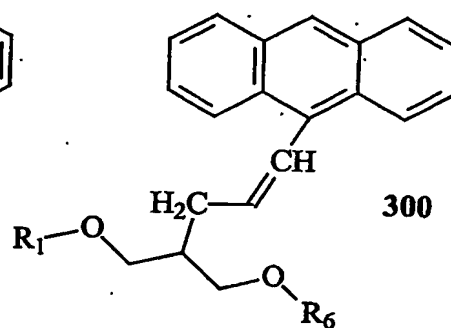
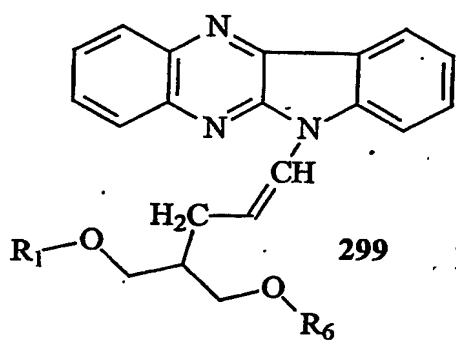
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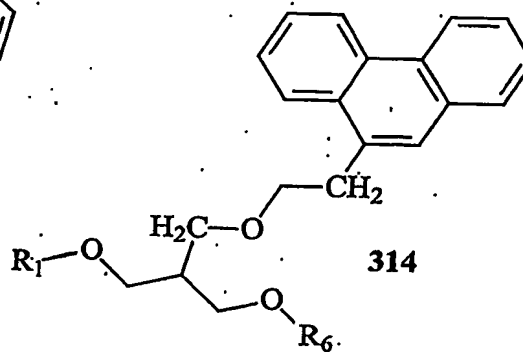
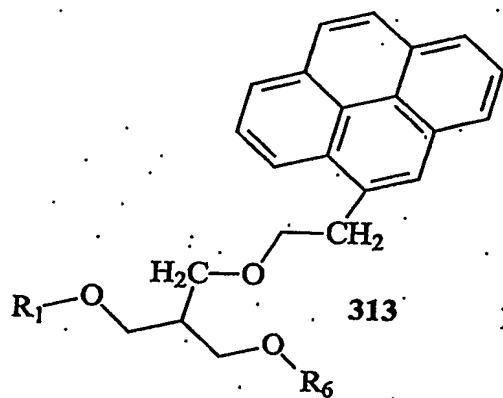
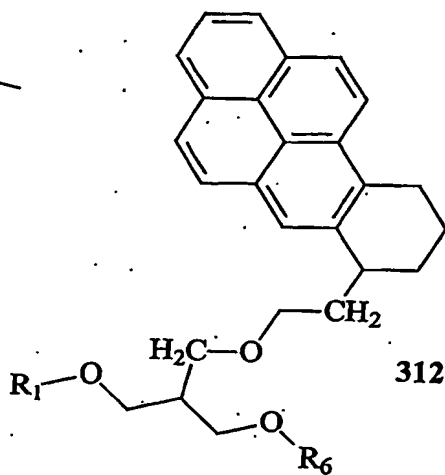
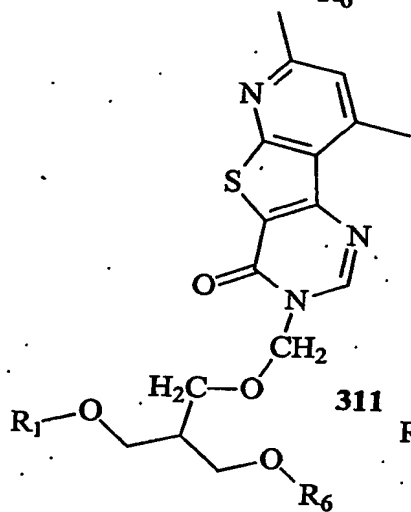
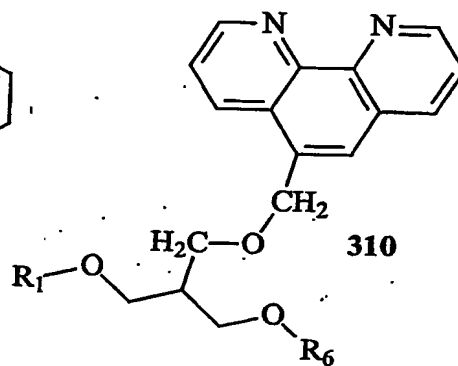
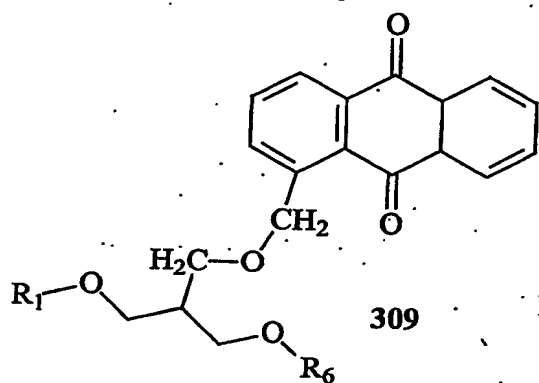
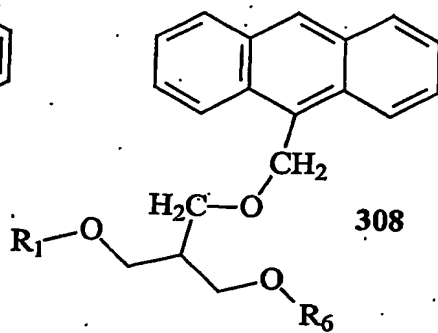
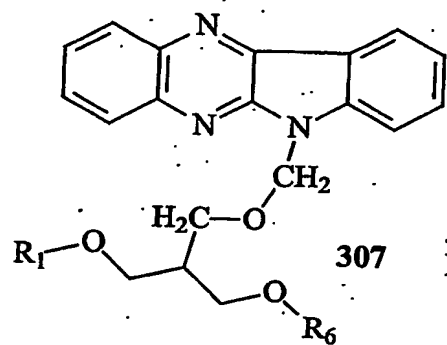


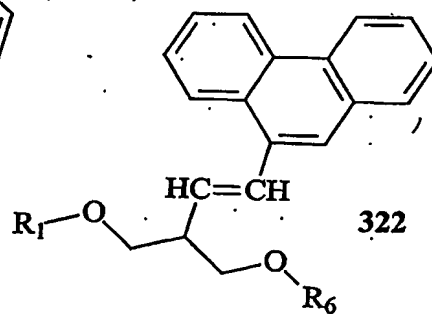
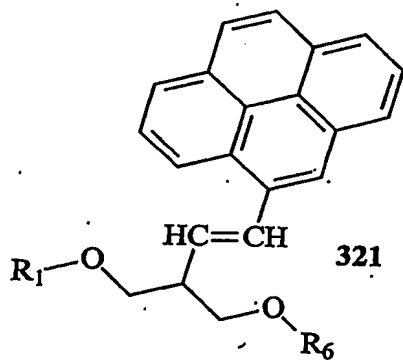
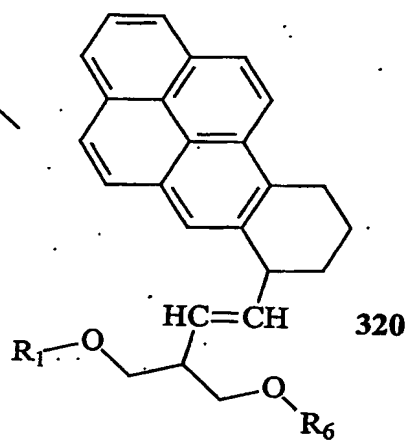
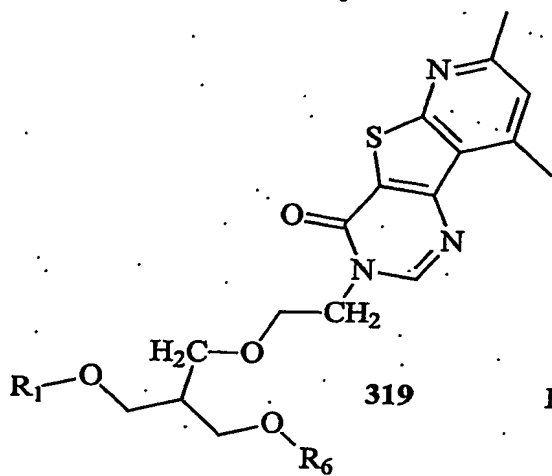
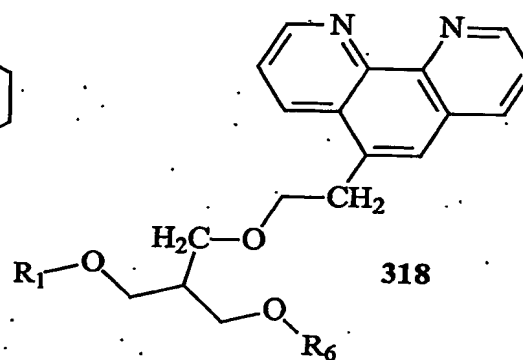
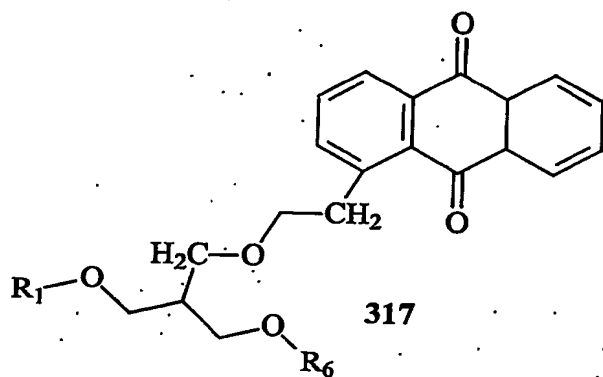
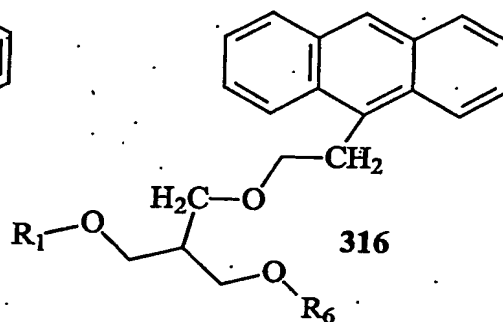
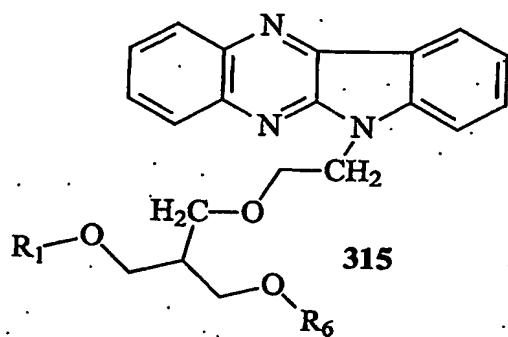


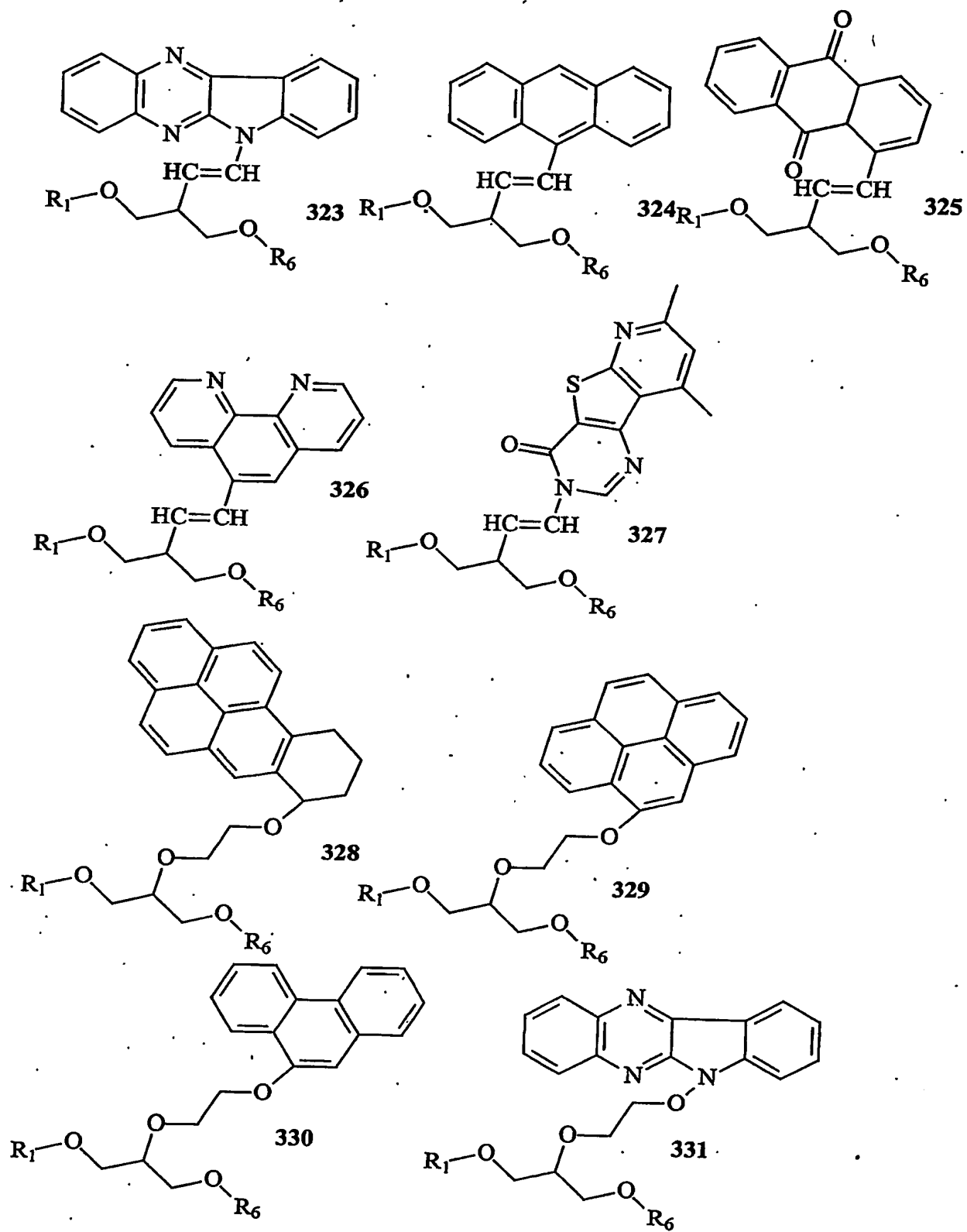


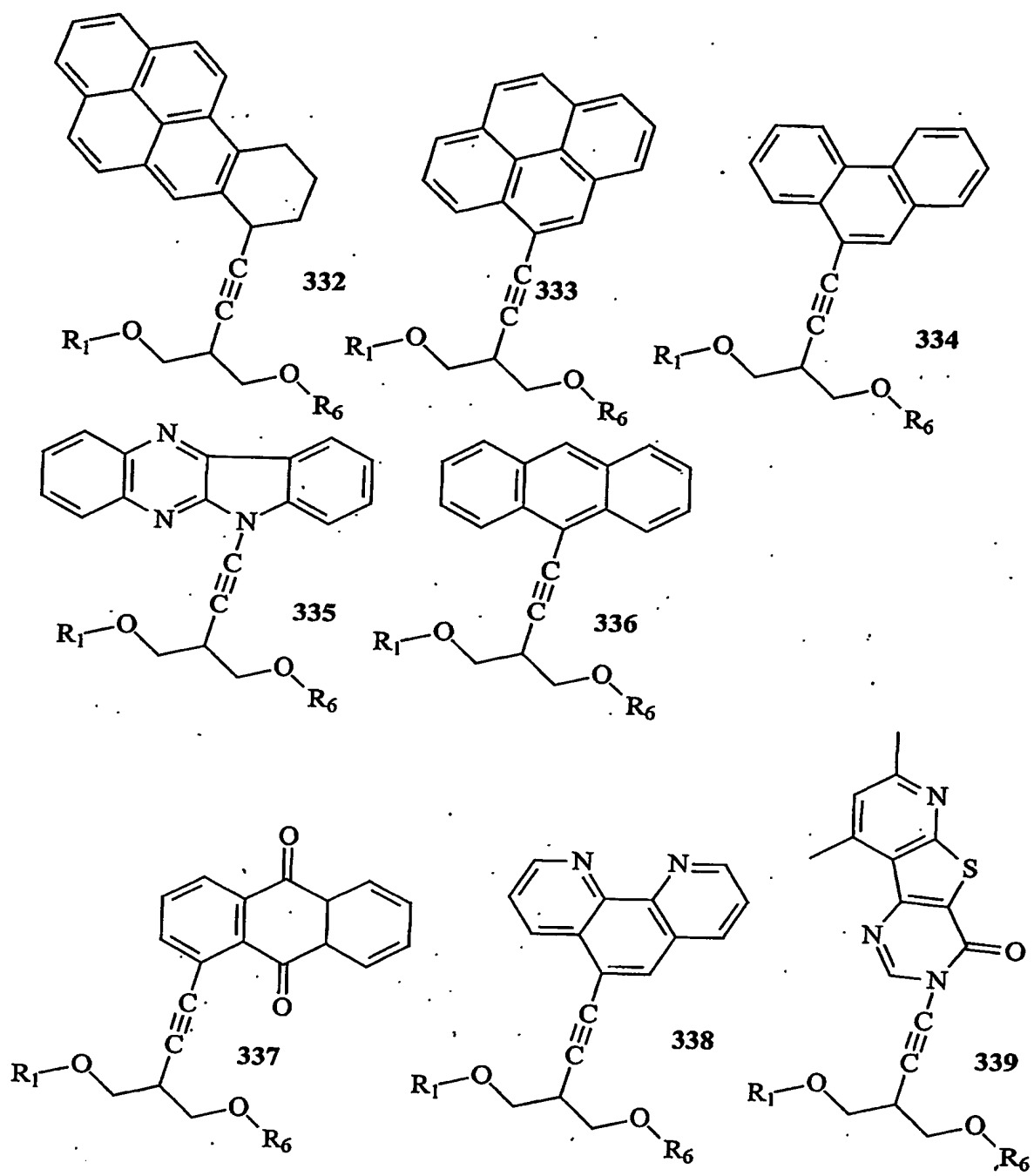












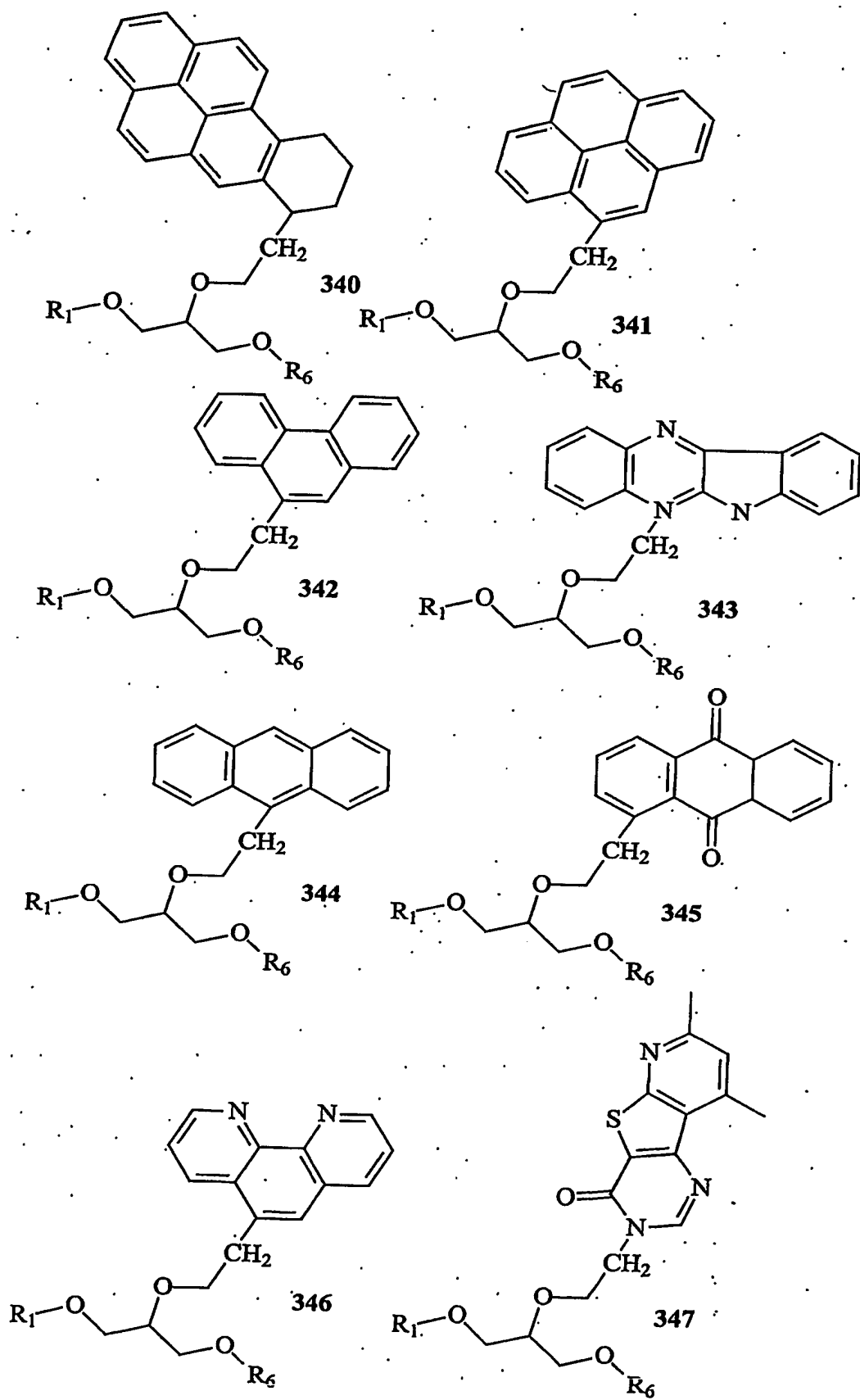


Figure 7. INA (intercalator pseudonucleotide) preparation scheme.

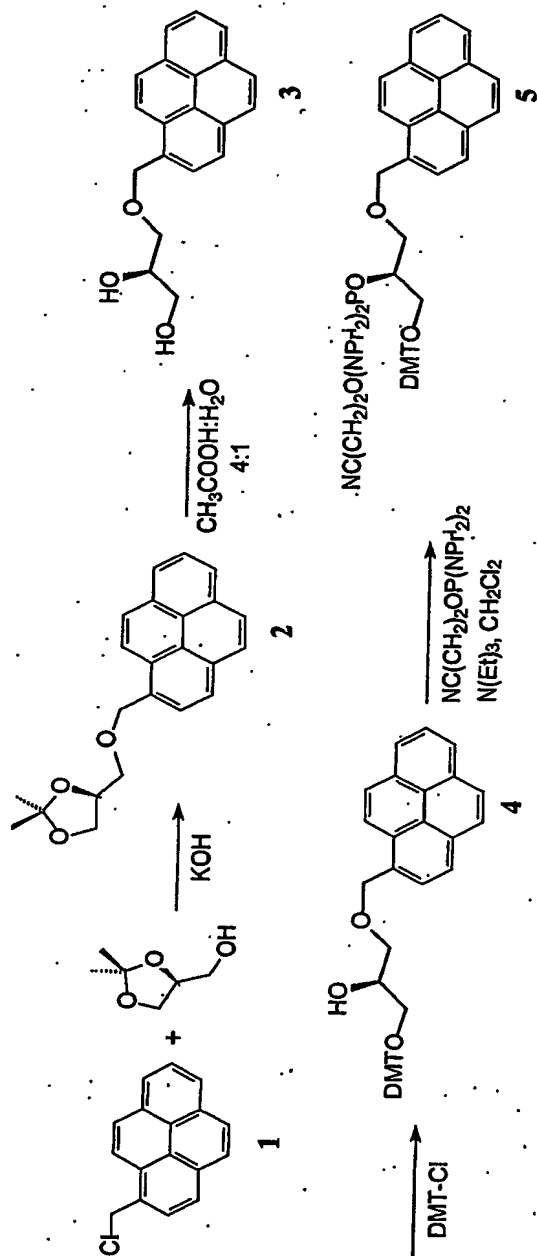
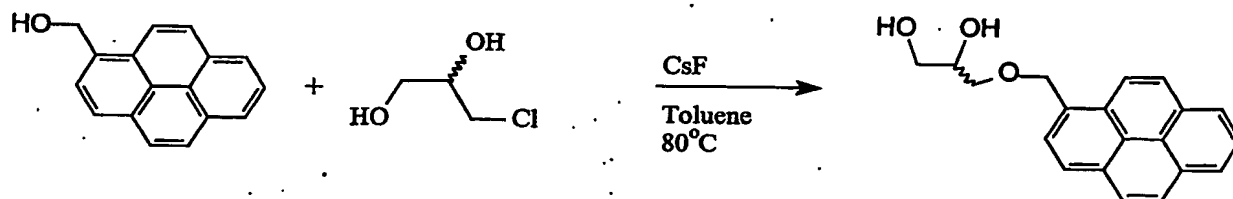
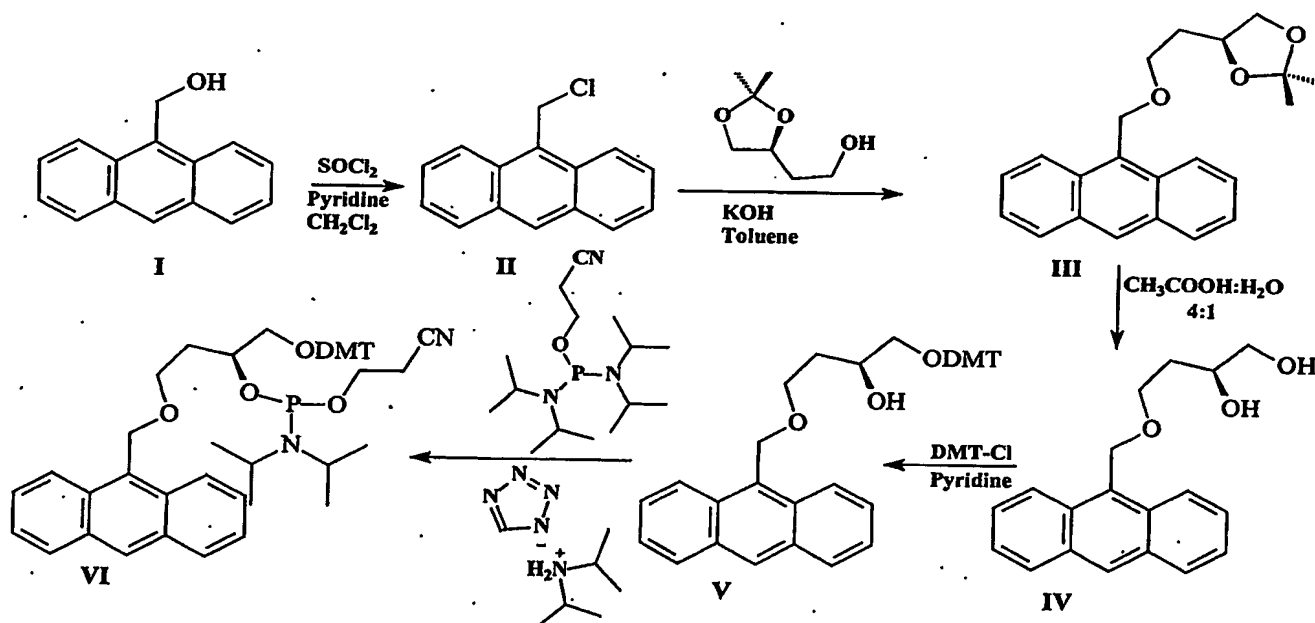


Figure 8



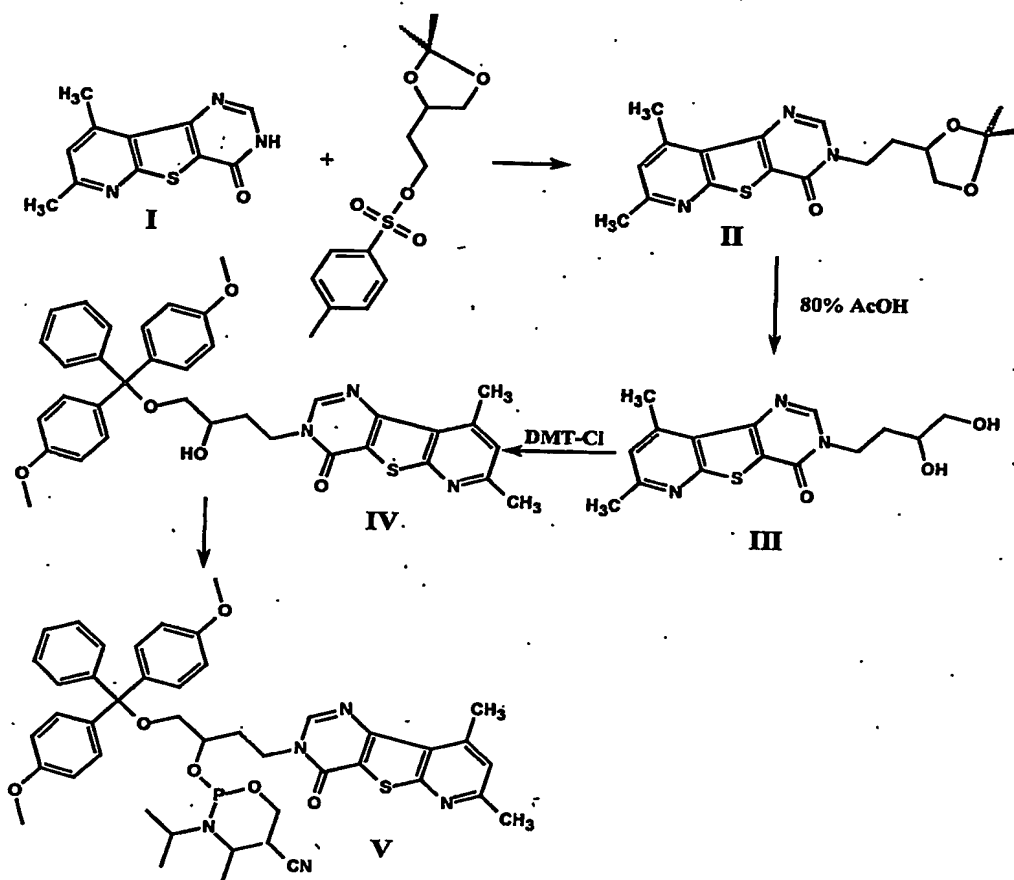
Scheme 1: Alternative synthesis procedure for 3-(1-Pyrenylmethoxy)-propane-1,2-diol

Figure 9



Scheme 2: Schematic view of the synthesis of the 2-O-phosphoramidite of 1-O-4,4'-dimethoxytrityl-4-O-(9-antracenylmethyl)-1,2,4-butanetriol

Figure 10

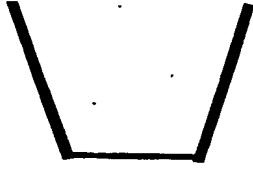


Scheme 3: Synthesis of the Phosphoramidite of (S)-1-(4,4'-dimethoxytriphenylmethoxy)-4-(7,9-dimethyl-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one)-2-butanol (V)


Figure 11

SandwichINA Signal Amplification


Methodology




N-Oxysuccinimide coated
Microtitre wells
+ Capture INA



Add Bisulphite treated
DNA



Wash



Add specific detection
INA /Fluorescent bead
conjugate




Quantitative Fluorescent
read-out for sample
detection

Applications

1. Cancer Diagnostics e.g Prostate.
2. Detection of single stranded DNA/RNA viruses e.g
3. mRNA Micro-arrays.
4. Mutational Analysis e.g genotyping.

Advantages

1. No PCR Amplification
2. Detection would be quantitative.
3. Extremely
4. Ease of automation.



Flourescent bead
+ Detection INA
+ EDAC

Figure 12 Sandwich INA signal amplification technology using magnetic beads

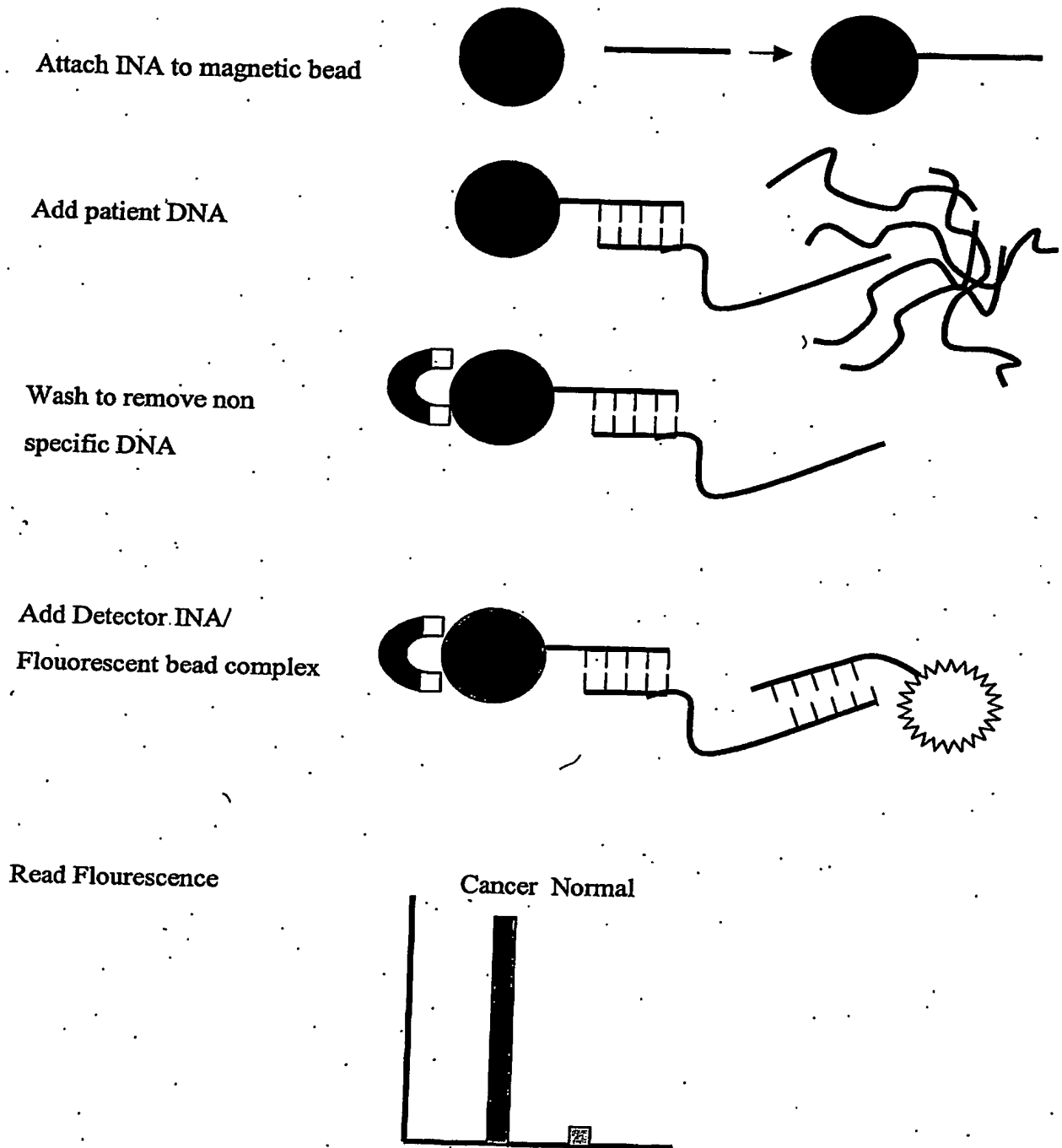


Figure 13

